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BRITISH JOURNAL OF PHARMACOLOGY AND CHEMOTHERAPY

EDITED FOR

THE BRITISH PHARMACOLOGICAL SOCIETY

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LONDON

BRITISH MEDICAL ASSOCIATION

TAVISTOCK SQUARE, W.C.1

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Brit. J. Pharmacol. (1949), **4**, 111.

SOME PHARMACOLOGICAL ACTIONS OF PIPERIDINE, PYRROLIDINE, AND OF PRESSOR CONCENTRATES FROM DOG URINE

BY

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(Received October 7, 1948)

Euler isolated piperidine from urine in 1944, and in 1945 (a and b) described its actions on blood pressure, respiration, rabbit's intestine, and the motility of the unanaesthetized frog. He indicated the general similarity of its action to that of nicotine. The pharmacological activity of the pyrrolidines has not been fully investigated. Tunnicliffe and Rosenheim (1902) reported that N-methylpyrrolidine (25 mg. i.v.) produced an initial fall of the cat's blood pressure followed by a rise, and that the response was not abolished by section of the vagi.

Lockett (1944, 1946) described the preparation of pressor concentrates from both normal dog and normal human urine, and referred to these as base B concentrates. After the incomplete oxidation of these concentrates with potassium permanganate, the following substances can be identified in pharmacologically significant amounts: piperidine, pyrrolidine, dimethylamine, and ammonia. Chemical evidence showing that these four bases were absent in the original concentrates, though they could be obtained as degradation products from base B concentrates, is to be presented elsewhere. The present communication is concerned with the equally important problem of comparing the pharmacological action of base B concentrates with that of the suspected degradation products. This is particularly of interest since the actions of pyrrolidine have been relatively little studied.

METHODS

Chemical methods

Preparation of base B concentrates.—The dog urine used for these experiments was brought to *p*H 11.0, and was submitted to continuous ether extraction for 24–48 hours in order to remove piperidine (Euler, 1945), nicotine compounds (Lockett, 1944), *iso*-amylamine (Bain, 1914), and other ether soluble material. The urine was then distilled in steam from highly alkaline solution, and the distillate concentrated *in vacuo* at a low temperature, without the addition of acid, as previously described (Lockett, 1946). That this procedure eliminated any piperidine or pyrrolidine which might initially have been present in the urine was shown by the disappearance of either base deliberately added to the urine in a concentration of 0.2 per cent.

Pyrrolidine was synthesized from trimethylene glycol: trimethylene dibromide was converted to the 3-bromopropylphenyl ether (v. Braun and Beschke, 1906) which was condensed with KCN. The nitrile was reduced with Na in alcohol and the phenoxy group replaced by iodine with hydriodic acid; the pyrrolidine was distilled from alkaline solution and converted into the picrate, m.p. 112° C. (Found: C, 40.2; H, 4.28; N, 18.5. Calc. for $C_4H_9N.C_6H_3O_7N_3$: C, 40.0; H, 4.0; N, 18.67 per cent.)

Piperidine was purified as the picrate by recrystallization. For the preparation of the hydrochloride the base was liberated from the purified picrate.

Dibenamine (N-N-dibenzyl-β-chloroethylamine) was obtained in solution in propylene glycol (50 mg./c.c.) by courtesy of Dr. A. Wilson.

Pharmacological methods

The techniques employed for the pressor test on cats under chloralose, for acute sympathectomy, adrenalectomy, and evisceration have been described (Lockett, 1946).

Collection of blood from adrenal veins.—The animal was prepared for blood pressure records and

for intravenous injections. Forelimb veins were exposed, and a midline abdominal incision was made; loose ligatures were placed in position. After 30 min. heparin was administered to the animal, and one or both adrenal veins were cannulated. The fine valve tubing which extended from the cannula led through a puncture incision in the abdominal wall, and returned the blood to a cannulated opposite forelimb vein. A very small glass T-piece interpolated into the tubing close to the abdominal wall was used for the collection of blood samples. The lumbar vein was tied as it approached the adrenal gland, and the abdomen was closed. The dead space from the cannula tip to the arm of the T-piece varied from 0.4 to 0.65 c.c.

Perfusion of the superior cervical ganglion.—The technique of dissection and the nature of the perfusion fluid followed the detailed description given by Feldberg and Gaddum (1934). The slightly pulsatile perfusion pressure varied from 125 to 130 mm. Hg, and the outflow was 0.7 to 0.9 c.c./min. The eserine concentration used in the perfusion fluid was 1:10,000. The effluent fluid was tested on the frog's rectus abdominis.

Survival sympathectomy.—Dogs were used. The sympathetic chains were removed from the stellate ganglia to the pelvis, in a 3-stage operation under cyclopropane, by a technique not greatly different from that described by Cannon *et al.* (1929). Sympathectomy was completed in 4–6 weeks. The tracings shown and the results quoted were obtained by aseptic cannulation of anastomotic branches of either femoral or brachial arteries, 3–7 weeks after the completion of sympathectomy.

Results

Six base B concentrates have been compared with solutions of piperidine and pyrrolidine in their actions on the blood pressure. Each concentrate was derived from 12 to 18 l. of dog urine, concentrated many hundredfold.

The relative activities of piperidine, pyrrolidine, and base B concentrates

In a series of 43 cats, under chloralose anaesthesia, and with mean carotid blood pressures between 110 and 130 mm. Hg, the sensitivity to both piperidine and pyrrolidine was found to vary greatly; the relative activity of pyrrolidine to piperidine, expressed in terms of base, varied from 2:1 to 0.6:1, pyrrolidine usually being slightly the more active. The intravenous injection of 1.5 mg. of either base into cats weighing from 2 to 4 kg. usually produced rises of blood pressure of 50 mm. Hg or more.

Since base B was examined in solution no comparison of activity could be made by weight. Piperidine and pyrrolidine are secondary amines and therefore give a red colour with β-naphthoquinone-4-sulphonic acid, a colour test which Euler (1945) used for the estimation of piperidine derived from normal urine. The pressor activity of three base B concentrates was too great to be explained in terms of their content of secondary amine, interpreted as piperidine and pyrrolidine, and determined colorimetrically, unless impurities were in part masking the colour reaction.

Whenever opportunity offered a comparison was made of the spontaneous alterations in the sensitivity of cats under chloralose anaesthesia to the intravenous injection of piperidine, pyrrolidine, base B concentrates, and adrenaline. In four cats in which sensitivity to adrenaline was unaltered or gradually increased in the course of two or three hours, sensitivity to pyrrolidine decreased greatly (e.g., 1:4); the sensitivity to piperidine also decreased (e.g., 1:2 or 2.5), whereas the response to base B concentrates was almost unaltered. Such a finding could only indicate that the pressor activity of base B concentrates was not due to their piperidine or pyrrolidine content: whatever the factors which contributed to such alterations in sensitivity, identical compounds should have been similarly affected.

Under rather deep chloralose anaesthesia, tachyphylaxis to pyrrolidine has frequently been observed in cats (Fig. 1), but it was not a constant finding. Such tachyphylaxis has not been demonstrated with piperidine or with base B concentrates.

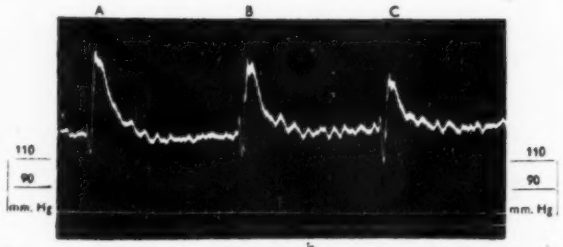

FIG. 1.—The tracings show the changes in the arterial blood pressure of a cat under chloralose anaesthesia following the injection of 5 mg. of pyrrolidine picrate (A), (B), and (C), at intervals of 5 min. Tachyphylaxis is seen. Time marker, 30 sec.

The influence of blocking agents

Interesting differences in the actions of piperidine, pyrrolidine, and base B concentrates were revealed after the use of autonomic blocking agents.

Tetraethylammonium chloride, given intravenously in a small dose (e.g., 10 mg./kg.) to six cats under chloralose anaesthesia, reduced, abolished, or reversed the pressor responses to piperidine and pyrrolidine, and caused only moderate reduction

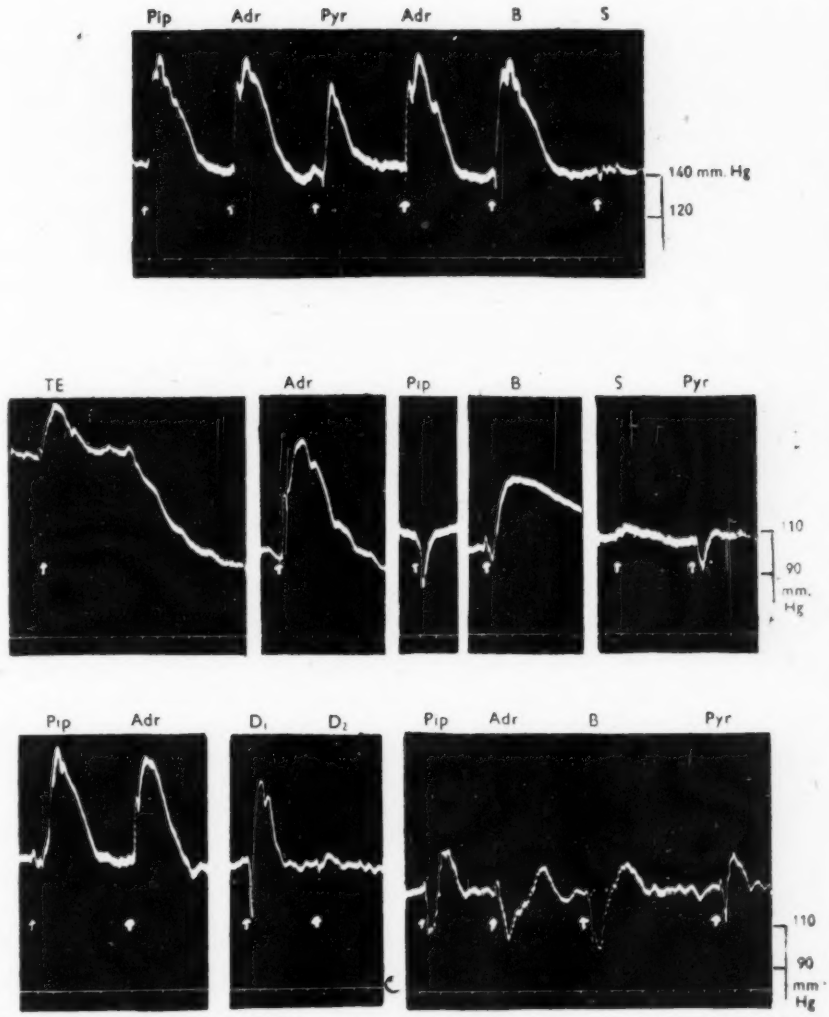

FIG. 2.—Cat, 2.7 kg., chloralose. Arterial blood pressure records; all injections intravenous. Top and middle records: Pip = 1.5 mg. piperidine HCl/8 c.c. Adr = 1.5 μg. adrenaline HCl/8 c.c. Pyr = 5 mg. pyrrolidine picrate/8 c.c. B = base B concentrate, 8 c.c. S = 8 c.c. normal saline. TE = 30 mg. tetraethylammonium chloride/10 c.c. saline. Pause of 10 min. made here. Bottom record: One hour after TE, when the effect of tetraethylammonium chloride had completely disappeared and the base line was restored to normal, the sensitivity of the cat to the compounds was unchanged; compare Pip and Adr with the first two injections of the top record. D_1 = 40 mg. dibenamine/0.8 c.c. propylene glycol, diluted to 10 c.c. with saline. D_2 = 0.8 c.c. propylene glycol similarly diluted. Note depressor responses after dibenamine.

in responses to base B concentrates (Fig. 2). Larger doses of tetraethylammonium chloride (e.g., 20 and 30 mg./kg.) reversed the responses to piperidine and pyrrolidine, whereas they never reversed but only reduced the response to base B concentrate.

Dibenamine was given intravenously, well diluted with saline, to 5 dogs under phenobarbitone anaesthesia and to 6 cats under chloralose anaesthesia, in doses varying from 5 to 20 mg./kg. Dibenamine was itself pressor. It reversed the responses to adrenaline more readily than those to piperidine or base B concentrates; the response to pyrrolidine was usually the least affected (Fig. 2). Reversed responses after dibenamine were not abolished by atropine sulphate (1 mg./kg.). The effect of dibenamine on the tachycardia produced after atropine in chloralosed cats by equipressor doses of piperidine, pyrrolidine, and adrenaline was, in the three experiments, a very similar percentage reduction of the maximum heart rate.

The effects of acute total sympathectomy

In cats under chloralose anaesthesia sympathectomy reduced the pressor response to piperidine and to pyrrolidine, whereas the pressor responses to base B concentrates and to adrenaline were little or not at all reduced. On two such occasions, despite the lowered base line after acute sympathectomy, almost as high a maximum value of the blood pressure was reached after as before sympathectomy. Evisceration reduced the responses to all three compounds.

The significance of the suprarenal glands

The sympathetic innervation of the suprarenal glands is composed of preganglionic fibres. Drugs which stimulate ganglion cells may therefore be expected to act directly on the medulla of these glands. In 3 cats under chloralose anaesthesia, a careful section of all nerve fibres at their points

of entry into the adrenal glands reduced the pressor responses to piperidine and pyrrolidine. These were not further reduced by subsequent adrenalectomy (Fig. 3). Such a finding was both contrary to expectation and to all the other evidence obtained, which had supported the view that these drugs have a stimulant action on ganglion cells.

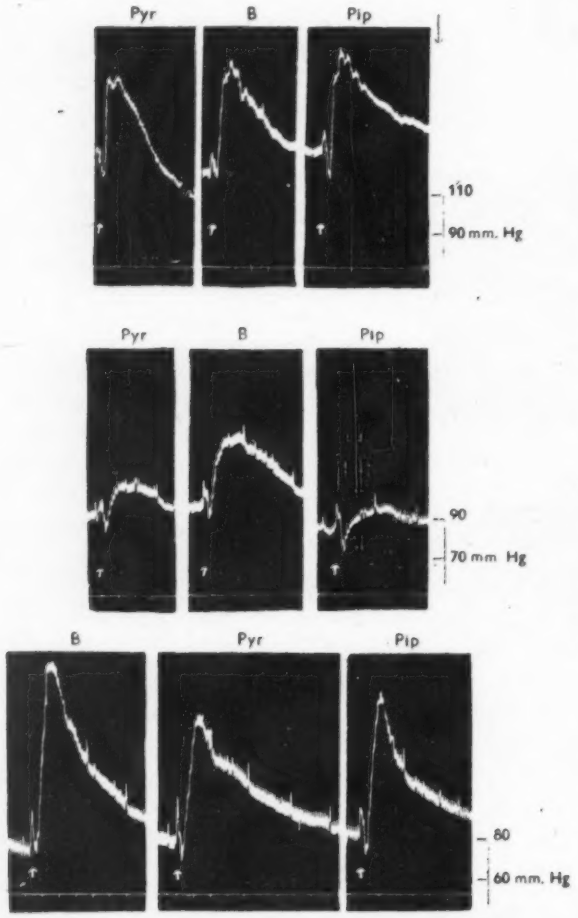

FIG. 3.—Cat, 2.7 kg., chloralose. Arterial blood pressure records. Top and middle records: Pyr = 4 mg. pyrrolidine picrate. B = 4 c.c. base B concentrate. Pip = 6 mg. piperidine picrate. Bilateral adrenalectomy at arrow. Bottom record: B = 6 c.c. base B concentrate. Pyr = 8 mg. pyrrolidine picrate. Pip = 12 mg. piperidine picrate. Time marker, 30 sec.

Two further experiments were carried out. Simple section of the splanchnic nerves did not alter the pressor responses which followed the intravenous injection of piperidine and pyrrolidine, but these responses were reduced by subsequent adrenalectomy. Paralysis of synaptic action by the intravenous injection of 40 mg. of nicotine, intravenously, in divided doses, into spinal cats, abolished the pressor responses to 4 mg. each of the bases, piperidine, pyrrolidine, and nicotine; the response to adrenaline remained. The results of these last two experiments conform with expectation: those of the previous experiment remain unexplained.

It was shown in two series of experiments that piperidine and pyrrolidine produce part of their pressor action by the liberation of adrenaline or another pressor compound from the adrenal glands.

In the first, blood was collected simultaneously from the femoral and adrenal veins, both before and during pressor responses. The blood samples were injected intravenously into another similar animal whose blood pressure was being simultaneously recorded. Six dogs and eight cats were used. Large pressor responses to piperidine and pyrrolidine were accompanied by the liberation of a pressor compound into the venous blood of a single adrenal gland which equated with 5 or 7 μg. of *dl*-adrenaline. The control adrenal and femoral blood samples were without pressor activity. Base B concentrates differed from piperidine and pyrrolidine; the adrenal venous blood collected during large pressor responses to base B concentrates was without pressor action on the blood pressure (Fig. 4). The quantities of adrenaline which appeared to be liberated from the adrenal glands during pressor responses to piperidine and pyrrolidine can be detected by chemical means.

In a second series of experiments, carried out on six cats under chloralose, samples of control adrenal vein blood and adrenal venous blood collected during pressor responses to piperidine and pyrrolidine were examined colorimetrically (Shaw, 1938). In two of the six experiments a well-marked blue colour was given by the control blood samples, indicating the continuous excretion of reducing compound; in the other four experiments the control blood yielded practically no colour. In all six experiments, a large increase in the reducing compound estimated in the adrenal venous blood occurred during the pressor responses to piperidine and pyrrolidine; piperidine usually caused the liberation of 1.5 to 1.3 times as much reducing compound as did pyrrolidine in equipressor doses. It should be stated that the amounts of reducing compound, estimated chemically as adrenaline, which were liberated from the adrenal glands by piperidine and pyrrolidine were, in all experiments, substantially less than half (e.g., 25–34 per cent) those which would account for the pressor responses obtained when such blood was

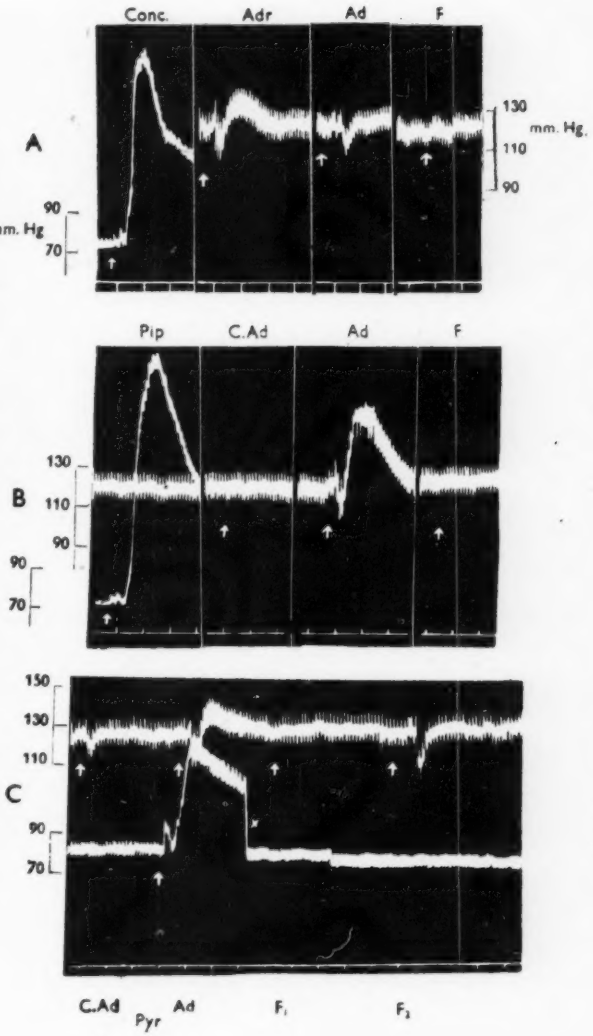

FIG. 4.—All but the first tracing in A and the upper tracings in B and C are records of the femoral blood pressure of dog 1, 7.6 kg., prepared for injections by femoral venous cannula; the other tracings are records of the femoral arterial pressure of dog 2, 8 kg., prepared by cannulation of both femoral and one adrenal vein for intravenous injections and the collection of femoral and adrenal venous blood samples.

A. Conc. = 20 c.c. base B concentrate to dog 2; during the response, blood was collected simultaneously from the femoral and adrenal veins. F = 5 c.c. of this femoral blood, and Ad = 3 c.c. of this venous blood injected into dog 1 without effect on the blood pressure. Adr = 5 μg. of adrenaline to dog 1.

B. Pip = 15 mg. piperidine HCl to dog 2. Whereas C.Ad = 4.0 c.c. control adrenal blood and F = 3.5 c.c. of femoral venous blood collected from dog 2 during the piperidine response (Pip) were without pressor effect, Ad = 3.5 c.c. of adrenal venous blood collected during the piperidine response had pressor action when injected into dog. 1.

C. The record shows the same procedure repeated with positive effect when dog 2 received 15 mg. pyrrolidine picrate (Pyr); C.Ad = 3 c.c. control adrenal blood; Ad = 3 c.c. adrenal and F_1 = 3 c.c., F_2 = 5 c.c. of femoral blood, all collected from dog 2 during the pyrrolidine response, and injected into dog 1. Time marker throughout 30 sec.

injected into another animal and was equated with *dl*-adrenaline.

Action in recovered sympathectomized dogs

The sympathetic chains of two dogs were removed from the stellate ganglia in the chest down to the pelvis, and after an interval of three weeks to allow time for the degeneration of the postganglionic fibres, the actions of piperidine and pyrrolidine were observed in eight experiments. Pyrrolidine and piperidine were found to have a depressant action on the blood pressure. Fig. 5 shows the responses of one such dog to 15 mg. pyrrolidine picrate and 10 mg. piperidine hydrochloride before and after survival sympathectomy. Tetraethylammonium bromide, when injected intravenously into two such preparations, failed to produce the customary fall in blood pressure which was certainly to be seen in one of these dogs before survival sympathectomy. Tetraethylammonium bromide did not abolish the depressor responses given in these preparations to piperidine and pyrrolidine, but 2 mg. atropine sulphate given intravenously reduced, and a further 2 mg. atropine sulphate abolished, the depressor reactions.

Cocaine, which prior to sympathectomy had not altered the pyrrolidine responses, and had slightly increased the piperidine responses, did not significantly increase the reversed responses to these two compounds encountered after sympathectomy (Fig. 6), which were also unaffected by dibenamine. Attempts to obliterate the action of dibenamine in these dogs by the use of sodium thiosulphate (Nomaguchi and Goodman, 1946)

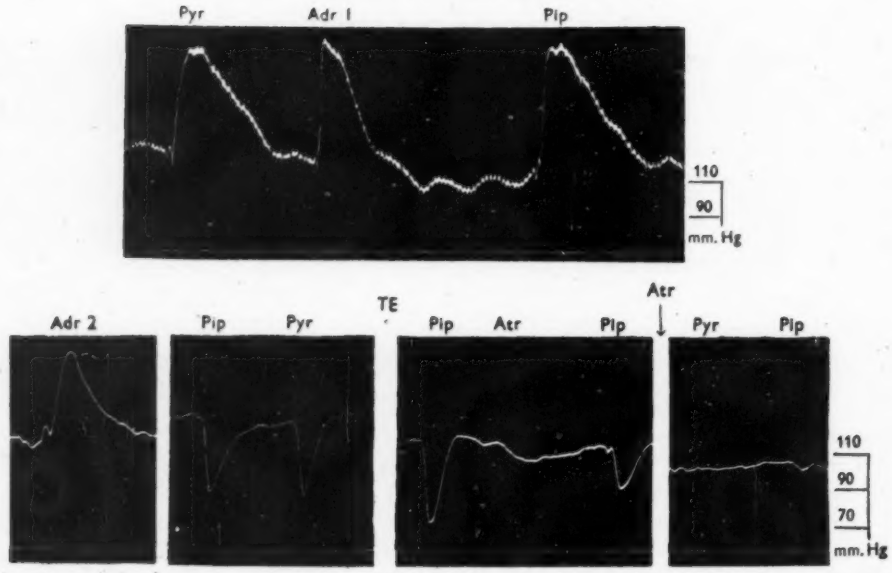

FIG. 5.—The tracings were taken under aseptic conditions from an anastomotic branch of the R. femoral artery of a dog, 7.5–7.7 kg., under phenobarbitone anaesthesia. The upper tracings were obtained before survival sympathectomy, and the lower were made five weeks after the completion of the last operative stage. Injections were given intravenously. Pyr = 15 mg. pyrrolidine picrate; Pip = 10 mg. piperidine HCl; Adr 1 = 20 μg. adrenaline chloride; Adr 2 = 4 μg. adrenaline chloride; Atr = 2 mg. atropine sulphate; TE = 100 mg. tetraethylammonium bromide in 20 c.c. normal saline. Time marker, 30 sec.

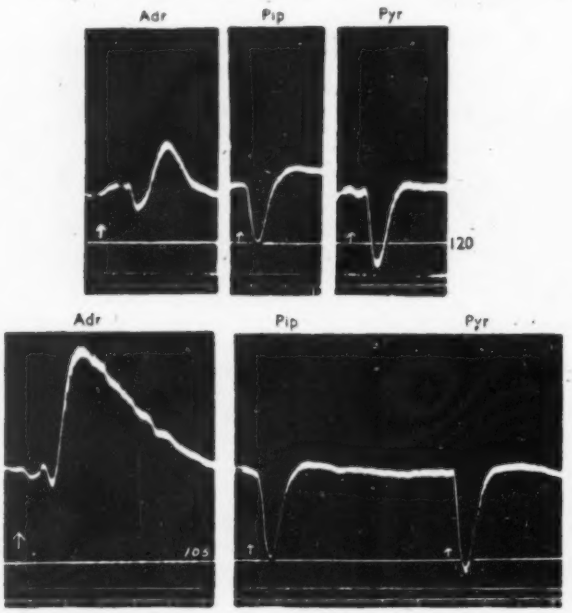

FIG. 6.—Dog, 7.6 kg., phenobarbitone. Record of the arterial blood pressure taken aseptically from a branch of the L. femoral artery, 19 days after the completion of survival sympathectomy. Intravenous injections: Adr = 2 μg. adrenaline HCl; Pip = 10 mg. piperidine HCl; Pyr = 15 mg. pyrrolidine picrate. Between the upper and lower tracings 30 mg. of cocaine HCl were given subcutaneously, and 40 min. were allowed to elapse. Time marker, 30 sec.

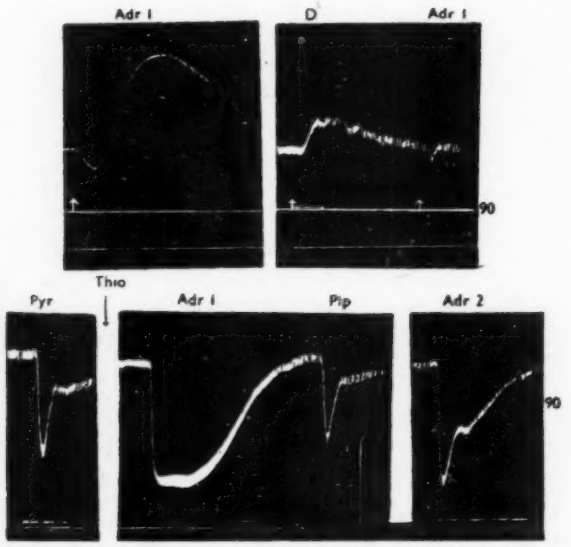

FIG. 7.—Dog, 7.7 kg., phenobarbitone. Aseptic record of the arterial blood pressure 46 days after the completion of survival sympathectomy. Intravenous injections: Adr 1 = 7 μg. adrenaline tartrate; D = 50 mg. dibenamine; Pyr = 15 mg. pyrrolidine picrate; Pip = 10 mg. piperidine HCl; Adr 2 = 2 μg. adrenaline acid tartrate. At arrow 20 c.c. of 10 per cent sodium thiosulphate injected slowly. Time marker, 30 sec.

resulted in a great potentiation of the adrenaline reversals, whilst the reversed responses to piperidine and pyrrolidine remained unaffected (Fig. 7).

Action on respiration

Euler (1945) described the stimulant action of piperidine on the respiration. The effect of piperidine, pyrrolidine, and base B concentrates on the respiration, when administered in equipressor doses, was studied in 5 cats under chloralose anaesthesia. Fig. 8 shows the great similarity of the respiratory changes after the intravenous injection of equipressor doses of piperidine and pyrrolidine; base B concentrate in an equipressor dose also stimulated respiration, but the effect was less dramatic and lasted longer (Fig. 8).

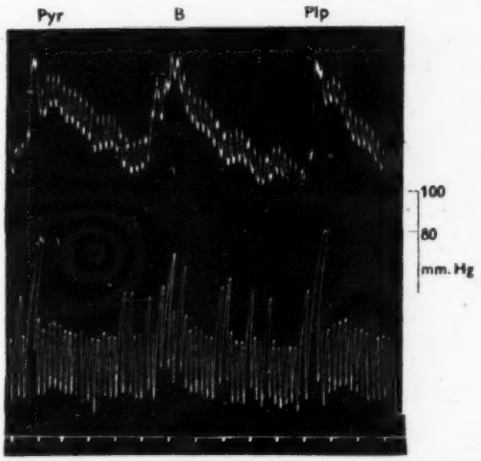

FIG. 8.—Cat, 3.2 kg., chloralose. The upper tracings are a record of the arterial blood pressure, the lower a simultaneous record of respiration. Intravenous injections: Pyr = 1 mg. pyrrolidine picrate; B = 3 c.c. base B concentrate; Pip = 0.75 mg. piperidine HCl. Time marker, 30 sec.

Action on sympathetic ganglia

It was found that although piperidine and pyrrolidine did stimulate the liberation of acetylcholine from the perfused superior cervical ganglion of the cat under chloralose, the dose required to produce this effect was very large in comparison with that which produced a considerable rise of blood pressure in the whole animal. Fig. 9 shows the responses of the arterial blood pressure of such a cat to the intravenous injection of 7 mg. pyrrolidine picrate, 5 mg. piperidine hydrochloride, and 2 c.c. base B concentrate (upper tracing). The lower tracing shows that approximately 1/10 of these pressor doses of piperidine and pyrrolidine when injected into the arterial cannula supplying the perfused cervical ganglion

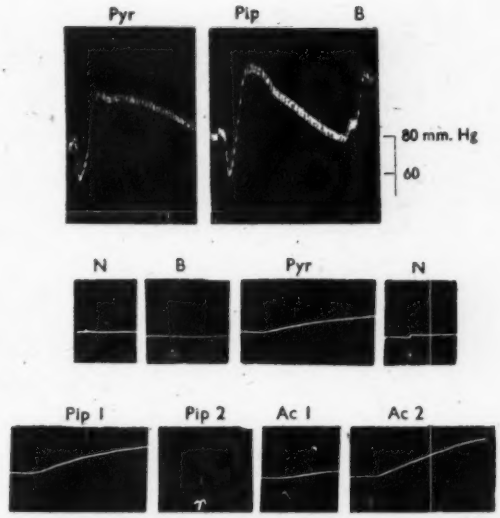

FIG. 9.—Upper tracing. Cat, 2.7 kg., chloralose. The record is of the arterial blood pressure; injections were intravenous. Pyr = 7 mg. pyrrolidine picrate; Pip = 5 mg. piperidine HCl; B = 2 c.c. base B concentrate. The R. superior cervical ganglion of this cat was being separately perfused; the perfusate contained 1 in 10,000 eserine.

Lower tracings show the responses of the eserinized frog rectus to 1.5 c.c. of the effluent perfusate, diluted 1 to 1.4; N = normal effluent; and after the following injections had been made into the S.C.G. cannula: B = 0.75 c.c. base B concentrate; Pyr = 1 mg. pyrrolidine picrate in 0.5 c.c.; Pip = 0.5 mg. piperidine HCl in 0.5 c.c. The remaining rectus responses follow the direct injection into the 15 c.c. bath of Pip 2 = 1 mg. piperidine HCl; Ac 1 = 0.25 c.c. and Ac 2 = 0.6 c.c. of a 1 in 1,000,000 solution of acetylcholine.

preparation in the same animal caused the liberation of acetylcholine into the effluent perfusate. Neither 1/10 nor 1/3 the pressor dose of base B concentrate similarly injected caused liberation of acetylcholine.

Action on uterus and intestines

Euler found that 0.5 mg. piperidine hydrochloride stimulated the contractions of rabbit's intestine, and that the effect produced was very similar to that of nicotine and was not abolished by atropine.

In four experiments piperidine and pyrrolidine were found to produce submaximal contraction of the guinea-pig ileum in a concentration of 10^{-4}, and this effect was prevented by atropine sulphate in a concentration of 5×10^{-4}. In three experiments 3×10^{-4} piperidine caused a greater increase in tone in the horns of a guinea-pig uterus than pyrrolidine did in the same concentration; each drug abolished the spontaneous activity and

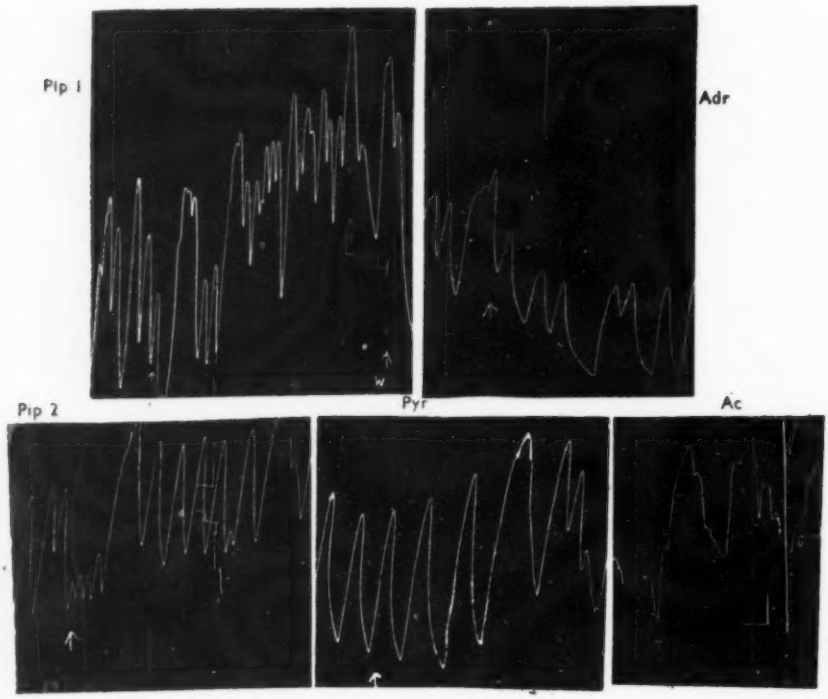

FIG. 10.—The record shows the responses of the non-pregnant cat uterus, suspended in oxygenated Tyrode solution, at 37° C., in a 20 c.c. bath, to the injection of the following solutions: Pip 1 = 1 mg. piperidine HCl; Adr = 10 μg. adrenaline chloride; Pip 2 = 0.5 mg. piperidine HCl; Pyr = 0.7 mg. pyrrolidine picrate; Ac = 0.5 mg. acetylcholine.

neither was effective in the presence of 5×10^{-4} atropine sulphate. (The concentrations given are those of the picrates.) In the non-pregnant cat uterus, whereas adrenaline caused inhibition, piperidine and pyrrolidine caused an increase in tone (Fig. 10), and were ineffective in the presence of atropine sulphate (5×10^{-4}).

Action on the nictitating membrane

Piperidine and pyrrolidine both caused contraction of the nictitating membrane in 4 cats and 2 dogs; the drugs were injected intravenously in doses sufficient to produce a rise in the mean arterial pressure of 40–60 mm. Hg.

DISCUSSION

Of the four basic degradation products obtained by partial oxidation of base B concentrates, piperidine and pyrrolidine exhibited pressor activity. The amounts of piperidine and pyrrolidine derived from base B concentrates have been approximately determined (Lockett, 1949) and might account for rather less than half the pressor action of the original base B concentrates, were it not for the dissimilarity in the actions of base B concentrates and these two bases. The main features of this dissimilarity are that the pressor action of base B concentrates is not reversed by tetraethylammonium chloride, and is only very slightly reduced after acute sympathectomy, denervation, or removal of the suprarenal glands. Piperidine and pyrrolidine cause the liberation of acetylcholine into the effluent perfusate from the superior cervical ganglion and of a pressor compound into the adrenal venous blood; base B concentrates do not, even when supramaximal pressor doses are employed.

These dissimilarities indicate that piperidine and pyrrolidine were not present as such in the original base B concentrates, although they can be obtained from base B concentrates by oxidative degradation, and that the pressor activity of these concentrates is due to some compound other than piperidine or pyrrolidine.

That piperidine and pyrrolidine have almost identical action is shown by the results recorded in this paper. Euler in 1945 described the action of piperidine as "synatropic," like that of nicotine, and commented on the absence of "muscarine effects." The presence of the latter in the action of piperidine and pyrrolidine has, however, been disclosed in experiments (i) on animals in which the postganglionic fibres of the sympathetic system have been allowed to degenerate, (ii) in perfusions of the superior cervical ganglion, (iii) on the non-pregnant cat uterus, and (iv) on the guinea-pig uterus and ileum.

SUMMARY

1. Piperidine and pyrrolidine have approximately equal pressor activity.

2. The pressor actions of piperidine and pyrrolidine were reversed by large doses of tetraethylammonium chloride, and by dibenamine, in cats and dogs (Fig. 2).

3. The pressor responses to piperidine and pyrrolidine were considerably reduced by acute total sympathectomy, and less markedly by removal of the adrenals or evisceration.

4. A pressor compound appeared in the adrenal venous blood during the pressor responses to piperidine and pyrrolidine (Fig. 4).

5. Pyrrolidine and piperidine produced depressor responses in dogs which had recovered from total sympathectomy. These responses were reduced by tetraethylammonium chloride, abolished by atropine, and unaffected by dibenamine and cocaine.

6. Pyrrolidine and piperidine stimulated respiration, caused contraction of the nictitating membrane and liberation of acetylcholine from the perfused cervical ganglion. Both compounds induced contraction of the guinea-pig ileum, increased the tone of the guinea-pig uterus and of the non-pregnant cat uterus ; these responses were antagonized by atropine.

7. The pharmacological actions of base B concentrates qualitatively resembled those of piperidine and pyrrolidine in that base B concentrates stimulated respiration and shortened the nictitating membrane. The pressor actions were reduced by dibenamine, by tetraethylammonium chloride in a dose of 10 mg./kg., and by evisceration.

8. The actions of base B concentrates differed from those of piperidine and pyrrolidine in that the pressor action of the former was not reversed by tetraethylammonium chloride 30 mg./kg., and that it was only very slightly reduced after acute sympathectomy, denervation, or removal of the suprarenal glands. No pressor compound appeared in the adrenal venous blood pressor responses to base B concentrates ; they also failed to liberate acetylcholine from the cat's superior cervical ganglion.

The greater part of the expense of this work was defrayed by a grant from the Medical Research Council.

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STUDIES ON THE METABOLISM AND MODE OF ACTION OF DDT

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(Received November 9, 1948)

Despite the world-wide employment of 2 : 2-bis (*p*-chlorophenyl) - 1 : 1 : 1-trichloroethane (DDT) as an insecticide, comparatively little interest has been shown in its mode of action and few investigations have been carried out on its metabolism, absorption, and excretion in contaminated animals. The present paper deals with the latter topics in an attempt to find out how DDT acts on living cells as well as on the organism as a whole.

METHODS

DDT and 2 : 2-bis(*p*-chlorophenyl)acetic acid (DDA) were estimated spectrophotometrically by the method of Schechter, Soloway, Hayes, and Haller (1945). Organically bound chlorine was determined simultaneously by the method of the writer (1948); urinary sulphur partitions by the volumetric benzidine method as modified by Elson, Goulden, and Warren (1945); urinary glycuronic acid by the method of Kapp (1940); urinary nitrogen, plasma protein, and non-protein nitrogen by the micro-Kjeldahl method; blood lactic acid by the method of Barker and Summerson (1941); glucose by the Hagedorn-Jensen method or by the technique of Nelson (1944); liver glycogen by the method of Good, Kramer, and Somogyi (1933); serum potassium by the method of Hoffman (1937); serum sodium by that of Kramer and Gittleman (1924) as modified by Rourke (1928); serum calcium, magnesium, and inorganic phosphate were estimated by the method of Briggs (1924), ascorbic acid being used as the reducing agent. Respiratory exchange *in vitro* was measured in the Warburg apparatus. Details of experiments with particular enzyme systems, etc., are given in the text.

Diphosphopyridine nucleotide (coenzyme 1) was prepared by the method of Sumner, Krishnan, and Sisler (1947); cytochrome C by that of Keilin and Hartree (1937).

The barium salt of adenosinetriphosphate (ATP) was obtained from Messrs. Boots, Nottingham, and purified as necessary.

Adenylic acid was obtained from Messrs. Light, Wraysbury.

Pure DDT (m.p. 108°) was administered in arachis oil in 10 per cent (w/v) solution, either orally by stomach tube or intraperitoneally by injection. The emulsion consisting of 1 part of the oily solution and 9 parts of a suspension of lecithin in 0.9 per cent NaCl solution, as recommended by Philips and Gilman (1946), was found to be satisfactory for intravenous injections.

DDA was used as the Na salt, prepared by the method of White and Sweeney (1945).

Most of the experiments were carried out on white rats and rabbits, but a few frogs were employed.

RESULTS

Tissue distribution of DDT

Table I shows the distribution of DDT and DDA in rats and rabbits after intraperitoneal, oral, and intravenous administration of 1,000 mg., 500 mg., and 50 mg. per kg. body weight respectively. Table II gives data on the recovery of DDT and DDA from the whole bodies of rats 3 hr. after intravenous injection of 50 mg. DDT/kg. Although the typical symptoms of DDT poisoning described by Cameron and Burgess (1945) appeared as a rule, there was some variation according to the route of administration: thus symptoms appeared within 6 hours of intraperitoneal injection, the animals becoming hyperexcitable, often displaying mild tremors for 48 hours but seldom going into convulsions; convulsions were the rule with oral and intravenous administration, after a latent period of 2 hours and 20 minutes respectively; death took place some 2–3 hours after the first convulsion.

From the results in Tables I and II it is evident that the only notable degradation product is DDA, and that this accumulates in tissues to a small extent. *p*-Chlorobenzoic acid was sought for, the analytical method used being the production of a rose-red colour by the dinitro compound under the conditions defined by Schechter *et al.* (1945): DDA is converted into tetranitrodichlorobenzophenone by intensive nitration, so that this neutral compound can be separated from dinitrochlorobenzoic acid after nitration of the

TABLE I

THE DISTRIBUTION OF DDT AND DDA IN RAT AND RABBIT TISSUES

(Rat tissues except where otherwise stated)

Tissue	Dosage	No. of observations	DDT mg./100 g. tissue			DDA mg./100 g. tissue		
			Mean	Range	S.D.	Mean	Range	S.D.
Liver	i.p. 1,000 mg./kg.	6	8.67	4.0–14.0	3.96	0.89	0.00–2.00	0.69
Kidney	,,	6	4.60	2.5–14.0	4.40	1.10	0.00–3.00	1.06
C.N.S.	,,	6	5.30	1.4–7.6	2.14	0.0	—	—
Muscle	,,	7	5.76	2.0–10.0	3.18	4.00	0.20–11.00	3.72
Heart	,,	1	4.80	—	—	0.0	—	—
Fat	,,	3	92.00	63.0–110.0	25.0	0.0	—	—
Blood	,,	6	3.40	1.7–5.6	1.70	0.0	—	—
C.N.S.	i.v. 50 mg./kg.	5	5.48	5.0–10.0	2.70	0.0	—	—
C.N.S. (rabbit)	,,	3	4.70	4.5–5.0	0.20	0.0	—	—
Cerebellum ..	,,	4	5.25	4.5–7.0	1.20	0.0	—	—
Heart	,,	5	7.60	2.0–20.5	7.30	0.0	—	—
Heart (rabbit) ..	,,	4	9.90	7.5–12.0	1.85	0.0	—	—
Muscle	,,	5	4.10	2.4–5.0	1.11	0.16	0.00–0.25	0.11
Blood	,,	5	11.60	9.0–15.0	2.46	0.0	—	—
Fat	,,	7	25.00	15.0–40.0	9.18	0.0	—	—
Liver	,,	10	12.80	6.0–20.0	5.09	0.0	—	—
Liver (rabbit) ..	,,	3	17.00	12.0–21.0	4.58	0.0	—	—
Kidney	,,	5	6.30	5.7–7.7	0.26	0.57	0.42–0.90	0.13
Kidney (rabbit)	,,	3	5.90	5.4–6.4	0.51	0.0	—	—
Adrenal (rabbit)	,,	6	20.00	17.0–25.0	2.96	0.0	—	—
C.N.S.	oral 500 mg./kg.	7	3.61	1.3–5.7	1.79	0.0	—	—
Liver	,,	6	7.96	7.0–9.0	0.84	0.0	—	—
Kidney	,,	6	10.60	8.0–15.0	2.05	0.0	—	—
Muscle	,,	7	6.00	3.0–9.0	3.09	1.43	0.00–4.80	2.07
Fat	,,	7	21.40	6.0–36.0	9.80	0.0	—	—
Blood	,,	5	1.50	0.8–2.0	0.44	0.0	—	—

alkali-soluble fraction ; no evidence of the formation of chlorobenzoic acid was obtained. The possibility that DDA might be decarboxylated to the corresponding ketone was also excluded. Since it has been suggested that DDT owes its toxic action to the liberation of HCl in tissues, evidence was sought that dehydrochlorination did take place. The product of this reaction, dichlorodiphenyl-dichloroethylene, yields dichlorobenzophenone on intensive nitration, which gives a red colour in the spectrophotometric test (Schechter *et al.*, 1945). If only part of the DDT were to react, a discrepancy between the value of alkali-labile Cl (Gunther, 1945 ; Judah, 1949) and the spectrophotometric determination would have arisen. No evidence for such a transformation could be obtained.

TABLE II

RECOVERY OF DDT AND DDA FROM WHOLE RATS INJECTED INTRAVENOUSLY WITH 4.6 MG. DDT/RAT

Rat No.	DDT mg.	DDA mg.
1	4.50	0.13
2	4.25	0.15
3	4.35	0.20
4	4.45	0.10
5	4.40	0.12
6	4.30	0.20

The Tables also show that there is great variation from animal to animal, even when the drug is injected intravenously, an observation which agrees with the findings of Smith and Stohlman (1945). Since some evidence exists that the symptoms of DDT poisoning are cerebellar in origin, estimations were made on the cerebrum and cerebellum separately in 5 animals. However, the concentrations of DDT were identical in both tissues, suggesting a uniform distribution of the compound throughout the central nervous system, with no special affinity of the cerebellum for the drug. Since Philips and Gilman (1946) found that ventricular fibrillation sometimes occurred in DDT intoxication, special attention was

directed to the concentration of DDT in the heart. The figures were not high, except in one rat which died within 20 minutes of an intravenous injection, presumably of ventricular fibrillation.

The amounts of DDT found in body fat were always high, an observation which confirms the finding of Woodward, Ofner, and Montgomery (1945).

Toxicity of DDA

Though DDA was seldom found in tissues after intravenous and oral administration of DDT, it was thought possible that this compound might be the toxic factor. However, rats can tolerate intravenous doses of 100 mg./kg. body weight of the acid, administered as the sodium salt. Estimations of amounts in tissues 18 hours after injection showed higher concentrations of DDA than were usual in acute DDT poisoning (Table III).

TABLE III

DDA IN RAT TISSUES 18 HOURS AFTER INTRAVENOUS INJECTION OF 100 MG. DDA/KG.

Tissue	DDA mg./100 g.			No. of observations
	Mean	Range	S.D.	
Liver ..	1.4	0.6–3.0	1.29	4
C.N.S. ..	0.4	0.0–0.75	0.33	4
Muscle ..	0.3	0.0–0.50	0.23	4
Kidney ..	1.6	1.0–2.50	0.76	4
Fat ..	—	—	—	4
Blood ..	2.0	0.3–3.25	1.05	4

Absorption and Excretion of DDT

Table IV gives some data about the absorption from the gut of DDT administered in nut-oil to 6 rats by stomach tube. The absorption rate is exceedingly slow, and it is paralleled by the excretion rate. Fig. 1 shows the excretion of DDT and DDA by rats after an intravenous dose of 2.5 mg. DDT. It can be calculated from these experiments that about 10 per cent of the total dose is excreted in 5 days. No DDT is excreted in the urine, which is in keeping with the observation of White and Sweeney (1945). A similar experiment on the excretion of DDT and DDA, after intraperitoneal injection of DDT, carried out on 12 rats showed that only 2.5 per cent of a dose of 1,000 mg./kg. body weight was excreted in 5 days. The total amounts excreted in this experiment were, of course, considerably higher than after intravenous injection.

TABLE IV

ABSORPTION OF DDT FROM THE GUT OF RATS

(3 hr. after administration by stomach tube)

Rat No.	DDT administered mg.	Found in gut mg.
1	72.0	64.0
2	90.0	80.4
3	125.0	103.0
4	125.0	90.0
5	125.0	95.0
6	125.0	91.0

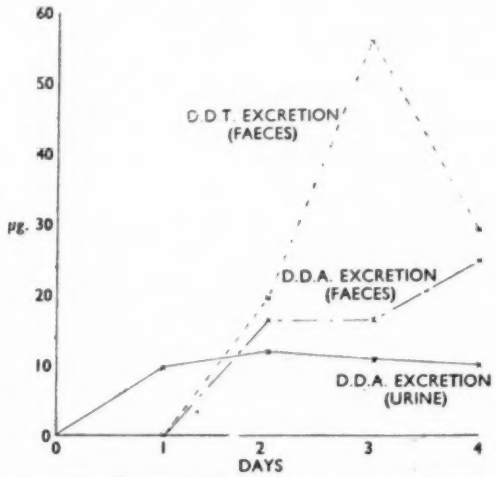

FIG. 1.—Faecal and urinary excretion of DDT and DDA by rats poisoned with 25 mg./kg. body weight of DDT i.v. Mean values for 6 rats.

Urinary chemistry in DDT intoxication

Since DDT is a halogenated hydrocarbon, the urinary sulphur partition and glycuronic acid excretion were studied in 12 rats after intraperitoneal injection of 1,000 mg. DDT/kg. body weight. The animals were kept on the standard diet described by Elson *et al.* (1945). No change attributable to DDT intoxication was observed in the excretion of sulphur or of glycuronic acid. Partition chromatography failed to disclose any alteration in the excretion of urinary amino-acids. Daily examination of the urine of these rats and of 6 others injected intravenously with 25 mg. DDT/kg. body weight gave no evidence of ketones, reducing substances, or of protein. Total urinary nitrogen increased abruptly in the group

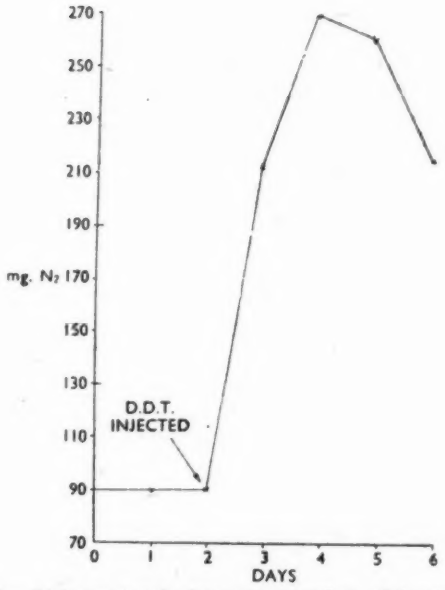

FIG. 2.—Urinary total nitrogen excretion of 6 rats given a dose of 25 mg./kg. body weight of DDT i.v.

injected intravenously (Fig. 2) and at the same time the rats lost weight, despite the fact that they were allowed food *ad libitum*; they were somewhat hyperexcitable and tremulous for the first 48 hours after injection.

Blood chemistry in DDT intoxication

The serum concentrations of potassium, calcium, sodium, magnesium, and inorganic phosphorus were determined in rabbits 2.5 hours after intravenous injection of a lethal dose of DDT. Relatively few animals were studied, since it was found difficult to obtain samples of blood when symptoms were well established. Of the 14 animals used, 8 provided samples which were so badly haemolysed as to be useless.

Table V illustrates the results. It is clear from these that the claim of Vaz *et al.* (1945) that DDT causes hypocalcaemia is groundless. The mean serum Ca was normal, but one animal showed the high value of 19.0 mg./100 ml. Cameron and Burgess (1945) also reported high serum Ca in some of their animals. The high serum K content may explain the cardiac irregularities described by Philips and Gilman (1946). During the acute phase in rabbits, auscultation of the chest revealed cardiac arrhythmias in nearly all animals. No objective method of recording the heart rhythm was available.

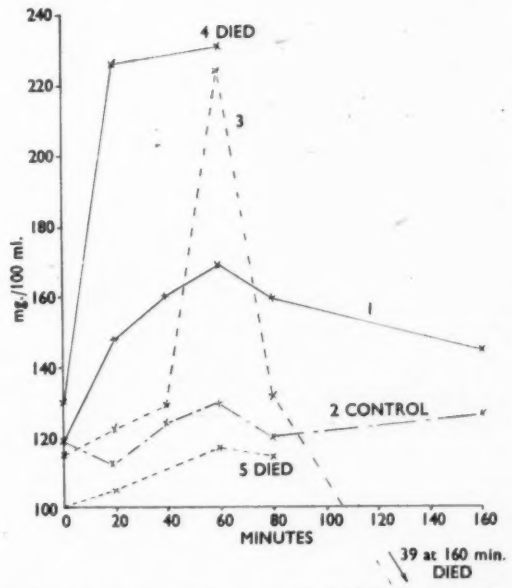

FIG. 3.—Blood sugar changes of rabbits poisoned with DDT. No. 2, control. No. 1, 25 mg./kg. DDT i.v. Nos. 3, 4, and 5, 50 mg./kg. DDT i.v.

Serum proteins were estimated in 6 rats receiving a lethal dose of DDT orally 2.5 hours after symptoms had started, but no change in protein concentration was observed.

The blood sugar and lactate were estimated in rats and rabbits during acute DDT intoxication. The results are presented in Figs. 3 and 4 and

TABLE V

SERUM ELECTROLYTES OF RABBITS POISONED WITH DDT (50 MG./KG. I.V.) AND KILLED 2½ HR. AFTER INJECTION

Substance	Control mg./100 ml. serum		Experimental mg./100 ml. serum		Number of observations	
	Mean	Range	Mean	Range	Control	Experimental
Sodium	320.0	316–320	328.0	321–336	4	6
Potassium	16.6	14.5– 20	45.0	20– 68	4	6
Calcium	14.0	14– 14.1	16.5	14.7– 19.8	4	6
Magnesium	2.5	2.0– 2.9	4.0	2.1– 6.0	4	6
Inorganic phosphorus	2.5	1.6– 3.3	4.0	2.5– 4.9	4	6

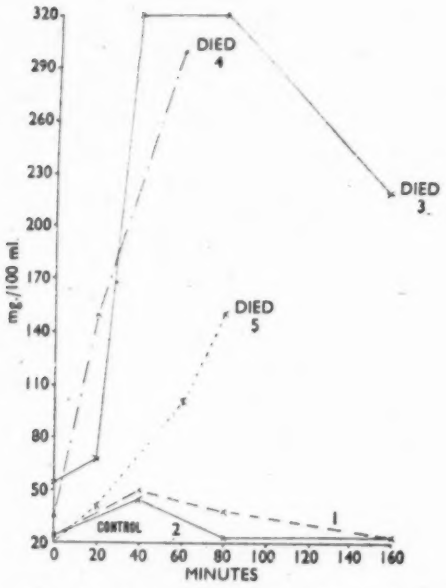

FIG. 4.—Blood lactate changes of rabbits poisoned with DDT. Numbering of curves identical with Fig. 3.

Table VI. The blood lactate rises abruptly in both species during the first hour of symptoms, while the blood sugar at first rises and then falls, sometimes to hypoglycaemic levels. However, this is neither the cause of DDT convulsions nor the explanation of death owing to DDT poisoning, for several animals maintained a normal blood sugar level, even 3 hours after a lethal dose, and despite pronounced convulsive seizures. Of course, it is possible that hypoglycaemia may contribute to a fatal termination in any given animal.

It is noteworthy that rats are far more resistant than rabbits to the derangement of blood sugar level.

TABLE VI

BLOOD SUGAR, LACTATE, AND LIVER GLYCOGEN OF RATS AFTER 50 MG./DDT/KG. I.V.

	Mean	Range	S.D.	No. of observations
	BLOOD SUGAR (mg./100 ml.)			
1st hr. experimental	124.3	114–141	12.0	6
1st hr. control	107.0	105–110	2.9	3
3rd hr. experimental	76.2	39–145	31.6	12
3rd hr. control	100.25	99–110	4.7	4
	LACTATE (mg./100 ml.)			
1st hr. experimental	72.0	53–100	22.2	6
1st hr. control	36.3	36– 37	0.4	3
3rd hr. experimental	74.3	36–100	27.0	12
3rd hr. control	29.8	27– 34	3.1	4
	LIVER GLYCOGEN (g./100 g.)			
3rd hr. experimental	0.2	0.00–0.40	0.2	5
3rd hr. control	3.9	3.0 –4.5	0.8	3

Liver damage and DDT intoxication

Liver glycogen was determined in 5 rats during acute DDT intoxication. After an intravenous injection of 50 mg./kg. body weight, the liver glycogen fell to vanishing point within 3 hours, at which time the animals were moribund (Table VI). It is interesting that, despite this, 3 of these animals had a nearly normal blood sugar level.

A further 6 animals (4 rabbits, 2 rats) were given the same dose of DDT and developed very severe symptoms and died within 0.5–1 hour. In each of these animals extensive, pre-existing liver damage of long standing was found on post mortem examination. In order to find out whether impairment of liver structure modifies the response of animals to DDT 12 rats were given subcutaneous injections of 0.2 ml. CCl_4/100 g. body weight, and this was followed 24 hours later with 50 mg. DDT/kg. intravenously. The effect was the opposite to that expected. All the CCl_4 group survived for at least 3 hours with only moderate symptoms, whereas the control group of an equal number of animals died within this time with severe symptoms. Liver damage was confirmed by autopsy and microscopical examination, and was assessed by Dr. K. K. Cheng as varying from 30–90 per cent of the total organ. To exclude the possibility of the trapping of DDT by necrotic liver cells, 4 of the livers were assayed for DDT. The content was uniform throughout healthy and damaged tissues and within the limits for normal animals receiving the same dose of DDT. No explanation can be offered for this paradoxical result.

The effect of calcium gluconate on DDT intoxication

Because Vaz *et al.* (1945) claimed that calcium gluconate prevents the symptoms of DDT poisoning, it was used in the treatment of rats given lethal doses of DDT. Three groups, each of 6 animals, were selected, together with an equal number of controls. In the first group, calcium gluconate (0.4 ml./100 g. body weight of a 10 per cent (w/v) solution) was injected intravenously 1.5 hours after 50 mg. DDT/kg. had been administered intravenously. No amelioration of symptoms was observed up to 1.5 hours after calcium

gluconate had been injected, but all the animals survived whereas the controls died. In the second group of rats, the same doses of calcium gluconate and DDT were used, but the former was injected a few minutes before the DDT. In this group, the animals developed only mild symptoms, and seemed almost normal 3 hours after injection, whereas the control rats were moribund at this time. The rats treated with calcium gluconate remained normal for the 48 hours during which they were observed. In the third group, calcium gluconate was administered, in the usual dose, before DDT, but the DDT dose was doubled. On this occasion no protective action was apparent. Both treated and untreated rats were dead within 90 minutes. No explanation of this calcium gluconate effect is obvious, but further reference will be made to it later, in connection with the effect of DDT on enzymes.

The effect of DDT on tissue respiration

Since the distribution experiments had given no information about the site and mode of action of DDT, preliminary experiments were made with slices of brain, liver, and kidney ; in addition, the effect of DDT on the respiration of rat diaphragm was investigated. Experiments were always run in duplicate. DDT was added from the side-arm of the Warburg flask after a preliminary control period of 20–40 minutes, to give final concentrations ranging from 0.001–0.0001 *M*. For these experiments, the emulsion used for intravenous injection was the best vehicle for adding the compound. When necessary, the emulsion was diluted with Krebs-Ringer solution. Controls were always carried out with the emulsion alone, but no effect due to it was ever recorded. The conditions of the experiments were those usual to tissue-slice work (Dixon, 1943).

TABLE VII

THE EFFECT OF DDT ON THE RESPIRATION OF TISSUES

Tissue	Conc. of DDT $\times 10^{-4}M$	No. of exp.	Percentage inhibition (−) or acceleration (+) of O_2 uptake	Percentage inhibition of anaerobic glycolysis
Liver	10.0	2	Nil	80
,, ...	5.0	4	Nil	75
,, ...	2.5	4	Nil	Nil
Brain cortex ..	10.0	2	Nil	Nil
,, ,, ..	5.0	2	Nil	Nil
,, ,, ..	2.5	2	Nil	Nil
,, ,, .. (without substrate)	1.0	2	+100	—
Brain cortex .. (without substrate)	0.5	2	+100	—
Kidney cortex ..	2.0	2	−20	—
,, ,, ..	1.0	2	−20	—
Diaphragm (rat)	10.0	4	+25	50
,, ,,	5.0	4	+19	30
,, ,,	2.5	2	Nil	Nil

Table VII shows the effect on the aerobic respiration and anaerobic glycolysis of liver, diaphragm, and brain cortex. Fig. 5 illustrates a typical experiment on the anaerobic glycolysis of muscle and liver ; the aerobic respiration of kidney cortex was also studied, but not its anaerobic glycolysis. The results show a very marked effect of DDT on the glycolysis of liver and muscle, but, paradoxically, not on that of brain. The aerobic respiration of all the tissues except kidney was little affected. In the latter, a 20 per cent inhibition was observed on the 3 occasions that kidney cortex slices were studied.

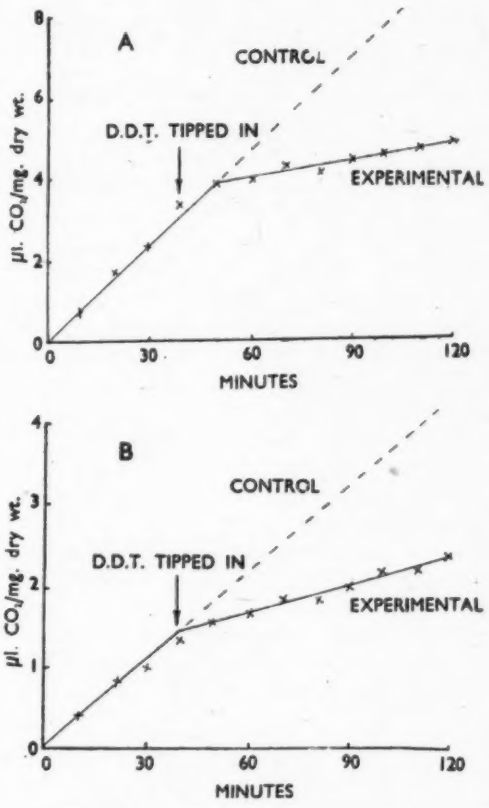

FIG. 5.—Effect of DDT ($10^{-3}M$) on anaerobic glycolysis of (A) liver slice, (B) rat diaphragm.

A curious effect was noticed when DDT in very low concentration (2×0.00001 *M*) was tested against brain and muscle in the absence of sub-

strate. Activations of oxygen uptake of up to 200 per cent were recorded. These experiments were not carried out on anaerobic glycolysis, and no explanation can be offered for them. The extra oxygen uptake was not due to oxidation of DDT. Assays on the content of the flasks revealed little breakdown. The latter problem also received attention during these experiments in the hope of finding the site and mechanism of DDT oxidation. Although experiments were carried on for 2.5 hours, little evidence of DDT breakdown was obtained. Indeed, with liver, diaphragm, kidney, and brain incubated with 100 μg. of DDT, 95 per cent of the compound could be recovered unchanged, and the remainder accounted for as DDA. Chemical estimations of the aerobic accumulation of lactate and pyruvate in liver slices and diaphragm showed that DDT had no effect on aerobic glycolysis.

Since DDT was found to exert a powerful effect on muscle and liver glycolysis, but none on that of brain, it was necessary to decide whether the absence of effect on the latter could be due to poor penetration of DDT into the brain slices; indeed, *in vivo* experiments suggest that brain always shows a poor concentration of DDT. In order to test this possibility, the effect of DDT on brain homogenates was investigated, since in these the fine state of division of the tissue should rule out permeability factors. Two types of homogenate were used, one prepared by the method of Elliott and Libet (1942), in which the tissue is homogenized in isotonic, calcium-free media at 37° C., and the other by the technique of Potter (1947) and Utter, Reiner, and Wood (1945), in which homogenization is carried out in ice-cold distilled water and the preparation fortified with ATP and coenzyme 1. The activity achieved by the latter type of homogenate is far greater than with slices. The former is not fortified, and has an activity comparable with that of cortex slices. DDT was found to have no effect on the oxygen uptake or anaerobic glycolysis of brain homogenates prepared by either method, and the results were confirmed for anaerobic glycolysis by chemical estimation of lactate produced in the Warburg flasks.

Despite these negative results, DDT was also tested against a variety of enzymes. DDT was without effect when tested against the following enzymes: aldolase (Dounce and Beyer, 1948); adenosine triphosphatase of brain and liver (Dubois and Potter, 1943); choline oxidase (Bernheim, 1940); glutamic acid dehydrogenase; glycolytic cycle in brain homogenates (Utter *et al.*, 1945); hexokinase; lactic dehydrogenase (Green and Brosteaux, 1936); malic dehydrogenase (Potter, 1946); pyruvic oxidase (Passmore *et al.*, 1933), succinoxidase (Schneider and Potter, 1943); triose phosphate dehydrogenase (Jandorff *et al.*, 1941); creatine phosphokinase (Lohmann enzyme) (Lehmann and Needham, 1937); aerobic phosphorylation (Loomis and Lipmann, 1948; Green *et al.*, 1948). In every test, the final concentration of added DDT was 5×10^{-4} *M*, except in that of the glycolytic cycle, where concentrations of 10^{-2} *M* and 10^{-3} *M* were also used; and in that of pyruvic oxidase, triosephosphate dehydrogenase, and the aerobic phosphorylation system, where concentrations of 10^{-3} *M* were also employed. The references cited give for each enzyme the methods used.

The system employed for oxidative phosphorylation requires further description. The "cyclophorase system" of Green, Loomis, and Auerbach (1948) was used according to the method of Loomis and Lipmann (1948), but in place of yeast hexokinase the transphosphorylation was accomplished through adenylic acid to fructose by means of a crude preparation of brain hexokinase (200 mg of acetone dried brain triturated with 4.0 ml. of ice-cold water and centrifuged; 0.1–0.2 ml. of the supernatant was added to each flask). This system esterifies inorganic phosphate at a brisk rate, but DDT did not affect either the oxygen uptake or the esterification. This experiment excludes the possibility that DDT might have an action similar to that of dinitrophenol in inhibiting synthetic reactions.

These findings did not rule out the possibility that DDT might act on a specific enzyme *in vivo*. Experiments were therefore undertaken to assay the activity of the glycolytic enzymes in tissues, and also to determine the activity of certain other systems.

Table VIII shows the results of such investigations. The same procedure was always followed: the animals were injected with DDT (50 mg./kg.) intravenously; 1 hour later they were killed and the enzyme assays carried out immediately. Controls were run in each experiment, and consisted of an equal number of animals, litter mates of the experimental group.

The assays gave surprisingly uniform results. Estimations of brain glycolysis (Meyerhof and Geliazkowa, 1947) in 6 rats agreed within 2 per cent, and showed no change between experimental and control animals, despite the fact that very severe symptoms were apparent in the poisoned

TABLE VIII

THE ASSAY OF ATP-ASE, AEROBIC RESPIRATION, AND ANAEROBIC GLYCOLYSIS IN RAT BRAIN AFTER ACUTE DDT INTOXICATION

	ATP-ase · μg. P liberated per mg. wet wt./15 min. (14 observations)		Aerobic respiration Q_{O_2} (30 min.) (6 observations)		Anaerobic glycolysis $Q^{N_2}_{O_2}$ (30 min.) (6 observations)	
	Mean	Range	Mean	Range	Mean	Range
Control	5.3	4.2–6.4	9.75	9.0–10.0	17.35	17.2–17.5
Experimental	4.95	3.4–6.4	9.0	8.0–10.0	17.5	16.6–18.0

animals. The agreement between the assays for ATP-ase was not so good. Dubois and Potter (1943) state that an experimental variation of 15 per cent may be encountered. No significant change was observed in a series of 12 animals. This enzyme was studied because it is known that it requires Ca ions for maximal activation *in vitro*, and it was hoped that this might provide an explanation of the calcium gluconate effect. Accordingly, each assay was conducted with and without Ca in the experimental vessels. In 3 rats poisoned with DDT it was found that, without Ca, only 30 per cent activity was obtained, whereas in the control group, both with and without Ca, the activity was maximal, and this was also true in the DDT group with Ca. This result could not be confirmed in a second experiment, carried out on 8 litter mates of the animals used in the first experiment. It must therefore be assumed to be fortuitous.

The aerobic respiration of the brain was assayed in a group of 6 rats. The experiment was carried out on isotonic homogenates prepared by the method of Elliott and Libet (1942), and, as before, negative results were obtained.

Discussion

Though a certain amount of information about the mode of action of DDT is available, considerable gaps still exist in our knowledge of fundamental points. Smith and Stohlman (1944, 1945) devised a method for the estimation of organically bound chlorine and applied it to the estimation of DDT in the tissues of several mammalian species. They also carried out pharmacological experiments with the compound. But their method lacks specificity, and they were unable to determine what fraction of the organic Cl was present in tissues as DDT and what as degradation products. White and Sweeney (1945) demonstrated that animals given DDT excreted in their urine an alkali-soluble compound which they identified as 2 : 2-bis(*p*-chlorophenyl)acetic acid (DDA) by *x*-ray diffraction studies and synthesis. Similar methods failed to disclose any unchanged DDT in the urine of their experimental animals. Stiff and Castillo (1945a, b) devised a method for the estimation of DDT, and applied it to the study of tissue distribution of the compound. Their conclusion that no DDT can be found in tissues may be criticized on the ground that during extraction of the tissues they carried out a saponification with alcoholic alkali, a procedure known to destroy DDT (Gunther, 1945). The degradation product formed by the interaction of DDT and alcoholic alkali (2 : 2-bis (*p*-chlorophenyl) - 1 : 1-dichloroethylene) does not yield a colour with their reagents (unpublished data). Schechter *et al.* (1945) published a specific method for the estimation of DDT and related compounds, depending on intensive nitration followed by the development of colours in benzene solution with methanolic sodium methylate, and subsequent spectrophotometric estimation. This method was later applied to animal tissues by Ofner and Calvery (1945) and by the present writer (unpublished). It is capable of estimating 10 μg. and upwards of DDT and DDA. The author devised a method for the estimation of microgramme amounts of organic chloride which makes it possible to carry out simultaneous determinations by both techniques on limited samples of tissue (Judah, 1949).

Several investigations have been carried out on the mode of action of DDT. Lauger, Pulver, and Montigel (1945) found that the blood sugar of animals exposed to the insecticide first rose and then slowly fell to hypoglycaemic levels. Simultaneously, the blood lactate rose and the CO_2 combining power of the blood fell to about 50 per cent of the normal value. The liver glycogen of rats was depleted within 3 hours of the administration of DDT. These authors maintained that this combination of events is the cause of death in DDT poisoning. On the other hand, Vaz, Pereira, and Malheiro (1945) claimed that DDT intoxication in dogs was relieved by the administration of calcium gluconate, and consequently they concluded that hypocalcaemia was the cause of the symptoms of DDT poisoning. No blood calcium data are included in their paper.

The action of DDT on enzymes has received little attention, although Richards and Cutkomp (1945) have shown that the drug is without effect on cholinesterase of brain, and Tobías, Kolros, and

Savit (1946) obtained no evidence that the acetylation of choline *in vitro* is affected by DDT. The latter authors demonstrated that whereas the acetylcholine content of insects in the paralytic stage of DDT poisoning is increased, this is not so in the frog and rat. Lewis and Richards (1945) tested the effect of DDT on tissue cultures, but could find no effect.

These negative results, together with the existence of a long latent period after the oral administration of DDT before toxic symptoms become apparent, suggest the possibility of degradation of DDT to a toxic compound in the body. Philips and Gilman (1946), using their preparation for intravenous injection, found that the latent period after this mode of administration was only 10 minutes. Hence they concluded that DDT is the toxic agent, but they give no estimations of amounts in tissues in support of their claim.

The results of the present investigation may be dealt with in two sections: (1) distribution experiments and (2) the physiological effects and mode of action.

From the former experiments it may be concluded that the main degradation product of DDT is DDA. It cannot be said to be the only one because (1) small amounts of another compound would not affect the recovery of the substance from the whole animal, the inherent error of the chemical estimation being of the order of 5 per cent; (2) agreement between chlorine determination and spectrophotometric data is subject to the same limit, and it is also possible that such a compound would be water-soluble and so escape analysis altogether. The only safe conclusion is that a toxic intermediate if formed must be active in very low concentration, but no evidence for its existence has yet been found.

The distribution figures also throw light on the theory of Martin and Wain (1944) that DDT acts by liberating HCl in tissues. Busvine (1945) studied this and other theories of the mode of action of the drug, and came to the conclusion that the ease with which a compound is dehydrochlorinated has no relation to its insecticidal activity. In the present work no evidence for the dehydrochlorination of DDT in tissues was obtained by direct analysis, and it may therefore be concluded that HCl liberation plays no part in the toxic action of DDT on mammals.

The investigation of the detoxication of DDT was a major part of the present work, and it was found that the breakdown of DDT both *in vivo* and by tissue slices *in vitro* is very slow. No evidence of breakdown by a specific tissue was obtained in either type of experiment; as a result of these findings, the problem of the mechanism of DDT oxidation was not further investigated.

If the rate of degradation of the insecticide is low, the excretion of DDT is exceedingly so. No evidence could be obtained of conjugated derivatives of DDA in the urine of rats, either with amino-acids or with glycuronic acid. This result is not surprising in view of the fact that rats excrete such minute amounts of DDA and DDT. Detoxication mechanisms for DDT must, therefore, be very inefficient. The storage of DDT in body fat may have a protective action, but it has not been possible to test this hypothesis.

The changes in blood chemistry after DDT poisoning are of some interest. Blood sugar and lactate levels of the type found have also been reported by Handler (1945) in a study of the effects *in vivo* of inhibitors of glycolysis. Unfortunately the results with DDT are complicated by convulsions in the experimental animals. These could account for the whole picture without any other hypothesis. If the experiments could be done on previously curarized animals the point could be settled, but this was impossible. Lauger *et al.* (1945) suggest that the action of DDT depends on the changes in blood sugar, lactate, and alkali-reserve already described. However, it has been shown in the present work that while the changes are qualitatively similar to those observed by Lauger *et al.*, they are not always quantitatively sufficient to cause death. The differences in the two investigations may lie in the rate of development of symptoms. The animals in Lauger's work were dosed orally and took about 24 hours to die, whereas in the present work intravenous injection was used for greater precision and death occurred within 3 hours; blood sugar and lactate levels were frequently nearly normal at this time; consequently hypoglycaemia cannot be accepted as a general mechanism for the production of symptoms or the cause of death in DDT poisoning.

The variations in the blood cations can probably also be attributed to the convulsions. Thus Heppel (1940) has shown that muscle during activity loses K, and this could account for the high serum K levels found in convulsing animals. The acidosis caused by lactate accumulation in the blood might result in the occasional high serum Ca values obtained. It should be mentioned that in a few analyses of tissues (muscle and brain) no significant variations in Ca and K levels could be found. The present work throws little new

light on the problem of treating accidental DDT poisoning, but three main points may be made.

(1) Glucose administration has been claimed by Lauger *et al.* to protect their animals from death; no attempt has been made to confirm this in the present work, but it may well be true in any given case. The liver glycogen data also support this view.

(2) Sodium phenobarbitone has been claimed by Philips and Gilman (1946) to protect animals from DDT poisoning. They suggest that the "specific" effect of this barbiturate on the motor cortex is the basis for this action. It would appear likely on *a priori* grounds that suppression of the convulsive episodes would have a beneficial effect. It should be remarked, however, that Philips and Gilman noted ventricular fibrillation in some of their animals despite the fact that they were curarized.

(3) Calcium gluconate has been shown to reduce the severity of DDT symptoms, when given in large doses. It is apparent, however, that protection is partial, and that the best results are obtained when calcium gluconate is administered before DDT. This takes away from its value as a means of treatment in accidental poisoning, but it should be worth a trial should such poisoning take place.

Experiments on the effect of DDT on enzymes and on tissue respiration have not elucidated the mode of action of the drug. It is difficult to explain the discrepancy between the action on liver and muscle, and that on brain cortex, especially as no effect could be demonstrated on the glycolytic enzymes in solution. It is possible that DDT is degraded to an active compound in liver and muscle, but recovery experiments performed on the contents of the Warburg flasks do not support this theory. The possibility of trace substances always exists. Against this possibility are the results of assaying the glycolysis and aerobic respiration of brain homogenates after DDT poisoning of the whole animal. The experiments of Philips and Gilman (1946) with eviscerated preparations support the view that DDT is not degraded in the liver or other visceral organs. These authors showed that the toxicity of DDT to eviscerated animals was as great or greater than to intact animals. A further possibility is suggested by the work of Pilgrim and Elvehjem (1945) on the effect of succinylsulphathiazole on enzyme assays in rat liver; they found that the drug exerted an indirect inhibitory effect on certain enzymes, and attributed this to a great accumulation of Ca in the tissues. It is conceivable that our techniques were such that analogous effects were nullified.

The mechanism of DDT action would not be solved, even by the discovery of a specific enzyme inhibition. The substance is so inert chemically that it is difficult to imagine any action due to it except by some such physico-chemical mechanism as an interference with cell-surfaces. There is some evidence for this possibility; thus Gordon and Welsh (1948) found that DDT causes repetitive firing of the isolated crayfish nerve and showed that the effect was partially reversed by Ca and Mg ions. Furthermore, Fathy (1948) claims that DDT causes expansion of a spread monolayer of myristic acid and cholesterol. These observations are suggestive, but it should be emphasized that loose reference to "surface action" by a drug does not help to elucidate the mode or the mechanism of its effect. Inert substances such as DDT may well have an indirect action on a specific enzyme system by so disorganizing the surface (or surfaces) of the intact cell that the access of co-factors or substrate to the system is disturbed. It may be impossible by present techniques to demonstrate this effect (1) because isolation of the enzyme involves destruction of cell structure or (2) because studies with intact slices permit only overall estimation of gas-exchange, etc., and the enzyme concerned may not contribute significantly to such changes. Advances in cytochemistry may provide means for investigation of such action.

Reference should be made to the evidence that DDT acts on the central nervous system and that symptoms are due to some central disturbance. The most convincing experiments are those of Philips and Gilman (1946), who showed that curarized animals injected with lethal doses of DDT developed typical convulsive episodes as shown by the electro-encephalogram. In addition, it has been found that frogs suffering from the typical symptoms of DDT intoxication lose the signs when the brain is destroyed, nor do they return when normal reflex activity is restored. If the cord transection is carried out before the injection of DDT, symptoms never arise.

In conclusion, it must be stated that the present investigation has failed to explain the mode of action of DDT, its most important objective. Furthermore, it has been impossible to prove or disprove the presence of a toxic intermediate during DDT metabolism by direct chemical estimation. The whole problem of liver damage pro-

duced by chronic DDT poisoning (Cameron and Burgess, 1945) remains unsolved; and the reason for the protective action of calcium gluconate is still to be explained.

Summary

1. The distribution of DDT in tissues and its absorption and excretion have been investigated.

2. Experiments on the metabolic fate of the drug indicate that DDA is the only metabolite. It is pointed out that small amounts of other substances may be produced. No conclusive evidence that DDT itself is the toxic agent can therefore be brought. Direct analysis shows that the HCl liberation theory is untenable in the mammalian species. It has also been found that DDA is relatively non-toxic.

3. Investigation of the blood chemistry in DDT intoxication shows that violent changes occur. The blood sugar rises and then tends to fall. Blood lactate rises to relatively high levels. These results are more pronounced in rabbits than in rats. The serum K of rabbits often presents high values; the serum Ca is never low, and is sometimes raised. Plasma protein, non-protein nitrogen, serum Na, Mg, and apparent inorganic P show no changes. Evidence is presented that the variations are probably secondary to the convulsive seizures suffered by animals poisoned with DDT.

4. Calcium gluconate affords a partial protection against DDT intoxication. It is shown that this is not because DDT causes a hypocalcaemia.

5. The relationship of liver damage to acute DDT intoxication was investigated. Though in some animals with long-standing, pre-existing liver damage DDT toxicity appears to be enhanced, with experimentally produced acute liver damage an opposite and paradoxical effect occurs. The problem of liver damage produced by chronic DDT poisoning remains unsolved. Liver glycogen is rapidly depleted in rats poisoned with a lethal dose of DDT.

6. Experiments with tissue slices and isolated rat diaphragm failed to show any important breakdown of DDT. Kidney, liver, brain, and intact diaphragm oxidized some 5 per cent of the DDT to DDA. The remaining 95 per cent was recovered unchanged. There appears to be no specific site for the breakdown of DDT.

7. Experiments on the respiration of tissues and on enzymes failed to show the mode of action of DDT. Inhibition of anaerobic glycolysis, which was found with liver and diaphragm, could not be demonstrated with brain cortex. DDT was also found to be without effect on the glycolytic enzymes in solution and on a variety of other enzymes. Some acceleration of aerobic respiration was observed with DDT in rat diaphragm and brain cortex slices (when the latter were exposed to the drug in the absence of substrate). The possibility that DDT might have an effect similar to dinitrophenol was shown to be untrue by the lack of effect of the drug on aerobic phosphorylation. Chemical estimation of pyruvic acid and lactate in the Warburg flasks showed that aerobic glycolysis was not increased in liver and muscle.

My thanks are due to Prof. G. R. Cameron, F.R.S., for much advice and encouragement; to Dr. C. H. Kellaway, F.R.S., for the hospitality of the Wellcome Foundation Laboratories where most of the work on enzymes was done; to Dr. H. H. Green, Ministry of Agriculture Veterinary Laboratory, Weybridge, for the determination of blood cations on some of the experimental animals; to Dr. C. E. Dent for carrying out chromatographic analyses on several urine samples; to Mr. H. G. Williams-Ashman and Dr. G. V. R. Born for much assistance and valuable suggestions; and to Messrs. A. Hemming, J. S. Pizey, and J. C. Godding for skilled technical assistance at different times during the investigation.

The writer is indebted to the Medical Research Council for a personal grant and to the Graham Research Fund of the University of London and the Agricultural Research Council for expenses grants.

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Brit. J. Pharmacol. (1949), 4, 132.

CONTRIBUTION ON THE THROMBOCYTOPENIC ACTION OF URETHANE IN DOGS*

BY

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(Received December 4, 1948)

Thrombocytopenic purpura in dogs has been obtained by subcutaneous urethane administration (Cruz and Moussatché, 1948). Suppurative reactions commonly occur in the skin of the experimental animals after a few injections. These reactions, possibly due to sensitization to the drug, are undesirable in experiments on the action of urethane. The present paper deals with the results of urethane administration by vein, a route much more suitable for controlling the toxic doses.

METHODS

Our routine haematological technique has been described in a previous paper (Cruz, Silva, and Mello, 1945). We have estimated bleeding time according Duke's method (1910), coagulation time by Lee and White's method (1913), and clot-retraction by Macfarlane's method (1939). Urethane was used as a 60 per cent solution in distilled water.

RESULTS

When urethane is administered in high doses (0.4 g. or higher per kg. body weight) the animals usually eliminate faeces and urine immediately after the injections and show somnolentia followed by sound sleep for 10–15 minutes. This slight anaesthetic effect is of short duration, and animals become normal again after a drowsy period of about half an hour.

No striking differences were observed between the action of urethane administered subcutaneously and intravenously so far as the time of the establishment of the purpuric picture or pathological findings are concerned.

The results of effective doses (0.4–0.5 g. per kg. body weight) administered to 5 dogs are shown in Table I. In 4 dogs platelets disappeared from the circulation between 6–9 days when bleeding time was already very much prolonged and the other signs of purpura were also present. The animals were sacrificed a few days later, and the haematological findings revealed a picture of severe anaemia. In one of these dogs (426–20) platelets obtained by fractioned centrifugation of 50 c.c. of normal dog's blood were injected daily soon after the urethane administration in order to observe any eventual interference in the pathological picture. This preliminary experiment was completely negative. In another dog (494–16) urethane was stopped after seven daily injections and blood changes were followed. Confirmation of other authors (Duke, 1911; Firket, 1922; and Tocantins, 1938) and our previous experiments (Silva and Cruz, 1945) concerning platelet regeneration have been verified. Normal platelet volume was attained 3–5 days after the beginning of the platelet regeneration, which only started 20 days after the absence of platelets in the peripheral blood. In one splenectomized dog (466–19) the evolution and the pathological picture were very similar to those of normal dogs treated with urethane (Table I).

In order to ascertain the optimal doses to obtain the picture of purpura, higher and smaller doses than those described have been tried. As higher doses, we tried eight intravenous injections, each 0.4 g. per kg. body weight, within a period of 36 hours (dog 437–3) as well as two daily injections of a slightly higher dose (0.5 g. per kg.) (dog 439–23). Both these animals died from acute urethane intoxication in 2–4 days before presenting any signs of purpura (Tables II and III).

A smaller dose was tried in dog 496–26 for a period of 45 days without any signs of purpura or other

* Aided by a grant from Dr. Guilherme Guinle.

TABLE I

Dog No.	Weight (kg.)	Dose of urethane per kg. (g.)	Day of experiment	Red blood cells (millions per cu. mm.)	Haemoglobin (g./100 c.c.)	Haematocrit (%)	Reticulocytes (%)	Leucocytes (thousands per cu. mm.)	Platelets (c.c. per 100 c.c.)	Bleeding time	Coagulation time	Observations	Post-mortem examination
425–10 ♀	4.6	0.4	0	5.0	10.65	35	0.5	10.5	0.40	3′ 25″	4′ 30″	Killed on 7th day	Few petechiae on intestine. Scarce petechiae on kidneys, lungs, and stomach.
			6*	—	5.0	—	—	—	0	25′	4′		
			7	1.8	3.8	12	0.1	10.2	0	35′	4′		
426–20 ♂	7.8	0.4	0	4.7	9.8	29	0.2	12.0	0.70	5′	4′ 30″	Killed on 10th day	Numerous petechiae on intestine and heart. Few petechiae on lungs, kidney (cortical zone), and skin. Bloody stools.
			4	3.0	5.6	19	0.1	10.0	0.50	4′	4′		
			9*	—	3.0	—	0.1	—	0	30′	4′		
			10	1.4	2.2	9	1.0	10.5	0	20′	5′		
462–22 ♀	5.6	0.4	0	—	9.2	—	0.5	8.8	0.35	3′	2′ 40″	Killed on 10th day. Bloody stools on 9th day	Numerous petechiae on small intestine and heart. Few petechiae on lung and kidney. Blood in the lumen of intestine.
			5	—	6.2	—	—	—	0.25	3′ 40″	4′		
			8*	2.4	4.5	16	0.1	1.4	0.15	3′	4′ 30″		
			9	2.0	4.0	14	0	1.1	0.01	30′	4′		
			10	1.8	3.8	13	0	0.2	0.01	35′	4′		
466–19 ♂ (Splenectomized 24 days before)	7.5	0.4	0	4.8	9.4	33	0.5	13.8	0.25	3′ 40″	5′	Killed on 10th day	Many petechiae on small intestine, lungs, kidney, stomach, heart, and urinary bladder. Scarce petechiae on skin.
			4	4.6	9.2	29	0.2	12.0	0.12	3′	5′		
			8	4.0	8.2	28	0.2	10.5	0.05	6′	4′ 30″		
			9*	—	7.4	—	—	—	0	30′	5′		
			11	2.5	5.0	18	0.1	10.8	0.01	60′	4′		
			15	1.0	1.8	7	0	1.8	0	60′	5′		
494–16 ♂	12.4	0.5	0	7.0	14.2	44	0.5	12.4	0.40	4′	3′ 40″	Died on 52nd day. From 7th until 12th days bloody stools	Scarce typical purpuric lesions on small intestine.
			4	6.8	14.2	46	0.2	12.0	0.40	4′	5′		
			7*	5.8	11.8	36	0.2	10.8	0.05	20′	5′		
			9	5.0	9.0	31	0.1	—	0	35′	4′		
			12	2.0	4.5	13	0.1	10.5	0.05	28′	4′		
			19	2.0	3.8	14	0.1	10.5	0.01	25′	4′		
			31	—	5.4	—	—	—	0.02	5′ 40″	6′		
			33	—	6.0	—	—	—	0.05	5′	4′		
			34	—	5.6	—	—	—	0.15	5′ 30″	4′ 30″		
			35	3.0	6.0	21	4.0	12.4	0.40	4′	5′		
			36	—	—	—	—	—	0.38	5′	4′		
			39	—	7.4	—	1.0	—	0.35	4′	4′		

* Urethane stopped

TABLE II

DOG 439–23♂ 9.4 KG.

Two intravenous injections of 0.5 g. urethane per kg. body weight daily (injections morning and afternoon)

Hours of exp.	Haemoglobin (g./100 c.c.)	Leucocytes (thousands per cu. mm.)	Platelets (c.c. per 100 c.c.)	Bleeding time	Coagulation time
0	13.2	16.2	0.35	4′	3′ 30″
31	13.4	—	0.35	3′ 35″	4′
55	13.0	11.6	0.38	4′	4′
81	14.2	10.8	0.36	5′	3′ 40″

NOTE.—Death at about 96 hr. After the first five injections, somnolentia for about 15–20 minutes. From the 6th injection, inappetence and the somnolentia became more prolonged. No blood in stools. At autopsy only great hyperaemia in intestines and central nervous system.

TABLE III

DOG 437–3♀ 7.4 KG.

Eight intravenous injections (each 0.4 g. per kg.) in the period of 36 hours

Hours of exp.	Haemoglobin (g./100 c.c.)	Platelets (c.c./100 c.c.)	Bleeding time	Coagulation time
0	11.40	0.40	5′	4′
3	11.10	0.35	6′	4′
7	12.80	0.42	4′	4′
10	12.80	—	5′	—
14	13.60	—	5′	4′ 30″
24	13.80	0.35	5′	4′
31	14.40	—	6′	—
37	14.40	0.42	5′	4′ 30″

NOTE.—Slight somnolentia, defaecation, and urination after the 1st injection. After the 2nd and 3rd injections somnolentia for about 15 minutes. After the 4th injection great excitation and sound sleep for about 2 hr.; after the 6th injection sound sleep. Bloody stools between 7th and 8th injections. Death around 45th hr. At autopsy conspicuous hyperaemia of intestinal mucosa. No purpuric lesions.

haematological manifestation. Appreciable loss of weight was observed in this animal (Table IV).

As we expected, the coagulation time was normal at all times in these dogs submitted to urethane. The bleeding time was inversely proportional to the platelet numbers. Bleeding times as high as 1 hr. 20 min. have been observed in severe cases. Clot-retraction was proportional to the platelets number. Skin fragility to mechanical trauma was observed in all animals after purpura had been established.

TABLE IV

DOG 496–26♂ 12.0 KG.

Daily administration of 0.2 per kg. intravenously for 45 days

Day of experiment	Haemoglobin (g./100 c.c.)	Leucocytes (thousands per c.c.)	Platelets (c.c. per 100 c.c.)	Bleeding time	Coagulation time
0	11.4	11.0	0.25	4′	5′
7	10.8	14.0	0.28	5′	4′
21	11.1	7.8	0.22	5′	4′
39	10.5	—	0.15	5′	4′ 30″
45	10.8	10.5	0.28	4′	3′ 40″

NOTE.—Urethane was not administered on 15th and 19th days of the experiment. Slight somnolentia of short duration after injection from 35th day on. Urethane stopped on 45th day.

Intestinal haemorrhages, manifested by the presence of blood in faeces, were very conspicuous during the period of haemoglobin drop, as has been described in the anaemia produced in dogs by oestradiol benzoate or anti-platelet serum (Cruz, Silva, and Mello, 1945).

SUMMARY

Administration of urethane by vein produces in dogs a similar purpura picture to that obtained with subcutaneous injections. For experimental investigation of the mechanism of purpura, intravenous injections are safer and more reliable because abscess formation, so commonly observed when the subcutaneous route is employed, does not occur.

The best dose of urethane for the production of purpura in dogs by intravenous administration is 0.4 g. per kg. body weight per day. Higher doses (1 g. per kg. body weight) killed the animals by acute intoxication in a few days before purpura was established. A smaller dose (0.2 g. per kg. body weight) did not produce purpura after daily administration for 45 days.

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A STUDY OF THE TOXICITY OF THE PROTAMINE, SALMINE*

BY

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(Received October 26, 1948)

The protamine, salmine, has become widely known in the combined form, protamine-insulin. It has been generally assumed that the protamines are non-toxic. Vartiainen and Marble (1941) found that the median lethal dose of salmine subcutaneously in mice and rabbits was 200–300 mg./kg. and concluded that it was non-toxic in ordinary doses—a view supported by its widespread clinical use with no allergic or other side-actions. Reiner, de Beer, and Green (1942) found an LD50 of 94 mg./kg. in mice with protamine (presumably salmine?). Ahlstrom and von Euler (1946) have reported essentially similar results with rats and guinea-pigs, using salmine and also the related protamines, clupein and scombrin.

A second possible clinical use of protamine is as an antidote for heparin, first suggested by Chargaff and Olsen (1937) and reported by Jorpes, Edman, and Thaning (1939). As the protamine for this purpose will probably be given intravenously, it raises the question of possible toxicity by this route. Thompson (1900) reported that the intravenous injection of the protamine, clupein, gave markedly toxic reactions in the narcotized dog, 15–18 mg./kg. killing the animals. The injection of smaller amounts of protamine caused a definite and relatively prompt fall in blood pressure, which returned to normal in 25–30 minutes. In addition to the fall in blood pressure, the respirations became markedly increased in rate and depth, and were followed by a period of apnoea. Thompson also noted a delayed clotting of the blood and a leucopenia.

Jappelli (1933) confirmed Thompson's results with salmine using the unanaesthetized dog, as did Jaques, Charles, and Best (1938); 5 mg. salmine sulphate per kg. caused a pronounced fall in blood pressure and dyspnoea. However, they found that this protamine had relatively little effect on guinea-pigs, and this led to the present investigations. Shelley, Hodgkins, and Visscher (1942) reported that intravenous or intracardiac injection of 120 mg./kg. of protamine into rats and guinea-pigs caused death in three minutes with typical anaphylactoid symptoms. The same dose subcutaneously caused death in approximately three hours. As indicated below these results of Shelley *et al.* are not in serious disagreement with those obtained by Jaques, Charles, and Best and by Vartiainen and Marble.

Materials and Methods

The protamine used was a sample of salmine sulphate supplied by the Connaught Medical Research Laboratories, University of Toronto. It was judged to be free from histamine as it had no effect on the isolated guinea-pig ileum. While the major part of the study has been conducted with one large sample of protamine, three other batches were used in the earlier experiments with the same results. The protamine was dissolved in saline in a concentration of 10 mg. per ml. For large doses 20 mg. per ml. solutions were used; these were somewhat milky, but, by drawing the solution up into the syringe and forcing it out a few times, a uniform suspension of the protein could be obtained. The protamine solution was injected into either the jugular or the femoral vein, and unless otherwise indicated the injection time was 11–14 seconds. Unless otherwise indicated, all animals were maintained under pentobarbitone anaesthesia.

Cell counts were made in an improved Neubauer haemocytometer. Platelets were counted using the citrate diluting fluid described by Fidlar and Waters (1941). Values for both red cells and platelets are given for the same fields counted. Correction of the platelet count for dilution errors by use of the red cell count was calculated, but, as this did not result in any significant change in values, it has been omitted in the values reported. Blood histamine was measured on the guinea-pig ileum after isolation by the method of Code (1937) and also by direct assay of plasma, the technique of Anrep, Barsoum, and Ibrahim (1947) being used. It has been established that this technique can be satisfactorily applied to plasma (Scroggie and Jaques, 1948). Identity tests for histamine were conducted on the blood pressure of the cat after administration of atropine. In certain experiments, glassware was treated with a silicone, General Electric Dri-Film No. 9987, as described by Jaques, Fidlar, Feldsted, and Macdonald (1946). The glycogen used was obtained from Eastman Kodak, Ltd.

* Read in part before the British Pharmacological Society, London, July 26, 1947.

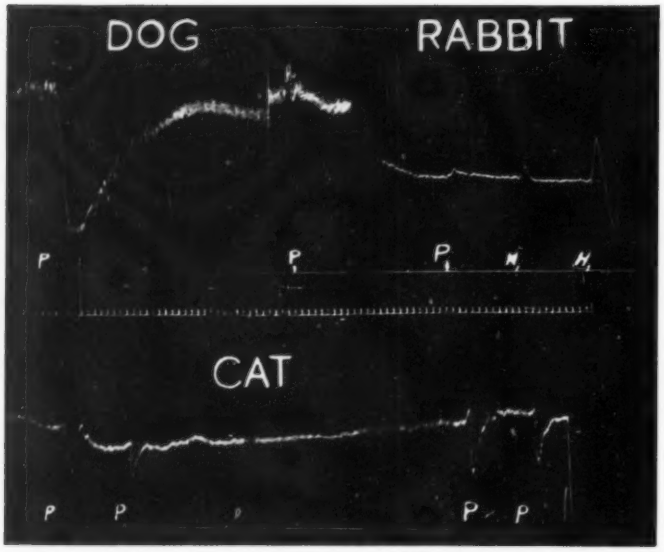

FIG. 1.—Effect of protamine on blood pressure. P = 5 mg. protamine/kg. intravenously. Time marker = 30 seconds. Dog: Initial B.P.—136 mm. Hg. B.P. started to fall after 45 seconds, minimum (30 mm. Hg) reached in 2 minutes 10 seconds; 3¼ hours elapsed between first and second injection. Rabbit: Initial B.P.—80 mm. Hg. H = 0.2 and 5 mg. of histamine. Cat: Initial B.P.—80 mm. Hg. Protamine at 3.05, 3.10, 3.36, and 3.41 p.m.

RESULTS

The effect of intravenous injection of protamine

In the previous study (Jaques, Charles, and Best, 1938) it was reported that the intravenous injection of protamine into normal dogs caused gastro-intestinal symptoms—retching, vomiting, diarrhoea, and cramps. In the anaesthetized dog, 5 mg. per kg. of protamine caused a marked fall in blood pressure. Associated with this was a marked hyperpnoea. These effects were also obtained with preparations from thymus (thymus histone) and from blood (globin) which, like protamine, precipitate insulin and neutralize heparin. In the present study, 5 mg./kg. of protamine was injected into animals under pento-barbitone anaesthesia and the blood pressure recorded (Fig. 1). Injection of protamine in the dog caused a very marked fall in blood pressure. A second injection three hours and fifteen minutes later had little effect on the blood pressure.

20 mg. of protamine were injected intravenously into a rabbit (2.8 kg.) under urethane anaesthesia (Fig. 1). There was a slight rise in pressure; 0.20 mg. of histamine caused a somewhat higher rise. This slight rise in pressure was not consistently obtained with protamine.

In contrast to the effects in the rabbit and dog, 5 mg./kg. of protamine injected intravenously in the cat caused a short sharp fall in blood pressure of 36 mm. Hg; this result was obtained each time the protamine was injected and hence did not resemble the reaction in the dog discussed in detail below.

Jaques, Charles, and Best (1938) previously reported that intravenous injection of protamine into guinea-pigs had no significant effect in doses up to 50 mg./kg. This is remarkable in view of the toxicity of protamine in the dog. In the present work guinea-pigs were given 50 mg./kg. of protamine intracardially. These animals showed mild anaphylactoid symptoms (snuffling, weakness of hind legs and then forelegs), but all recovered in an hour, with no deaths. Similar reactions were obtained in a series of guinea-pigs given 100 mg. of dialysed Witte peptone intravenously. It appears likely that this is the earlier stage of the effect observed by Shelley, Hodgkins, and Visscher (1942), who established that 120 mg./kg. would be lethal in three minutes in the guinea-pig. As discussed later, this toxicity for the guinea-pig at dosage levels ten times that in the dog may be due to a quite different mechanism.

FIG. 2.—Effect of protamine on blood pressure and respiration in the dog. Protamine—5 mg./kg. Time marker—15-second intervals. Initial B.P.=134 mm. Hg.

The toxicity of protamine intravenously in the dog

Since protamine is most toxic for the dog, further studies were conducted on this animal. The immediate effect of 5 mg./kg. of salmine intravenously on blood pressure and respiration of the dog is shown in more detail in Fig. 2. There is a slight hyperpnoea followed by a period of apnoea, which is then succeeded by normal respiration. The protamine preparations did not give the marked dyspnoea previously reported by Thompson (1900) with thymus histone and clupein. The apnoea appears with the initial hypotension. After a lag of thirty seconds, the blood pressure quickly falls to a minimum (30 mm. Hg), then recovers, reaching a level about 10 mm. Hg below the initial blood pressure in six to ten minutes. The initial blood pressure is not completely attained for an hour or more, but the period of hypotension has been measured only to the point where the blood pressure tracing became level again.

The effect of various dose levels of salmine administered intravenously in the dog is reported in Table I: 0.1 and 0.5 mg./kg. had no effect on blood pressure although there was a slight effect on respiration; 1.0 mg./kg. appeared to be the minimal effective dose and it caused a transient fall in blood pressure. Doses of 5 and 10 mg./kg. gave the typical fall in blood pressure of short duration already described. With doses of 15, 20, and 35 mg./kg. the hypotension was of longer duration, but this was due to the slowness of complete recovery; marked hypotension persisted for a much shorter time (eight minutes with a 35-mg. dose), and the blood pressure reached half its initial value in 15 minutes (15 mg.), 5 minutes (20 mg.), and 70 minutes (35 mg.). Even with the highest dose (35 mg.) the animal recovered.

TABLE I

EFFECT OF PROTAMINE INTRAVENOUSLY IN THE DOG

Dog	Dose mg./kg.	Ori inal blood pressure	Maximal fall in B.P.	Duration of hypo-tension	Change in respiration
C1	0.1	120	None	—	Shallow and rapid
C10	0.5	98	,,	—	—
C29	1.0	130	24	1′	—
C13	5.0	100	80	6½′	Cheyne-Stokes
C22	5.0	120	60	6′	Dyspnoea
C29	10.0	144	140	8′	Apnoea
C28	15.0	86	86	40′	Apnoea
C1	20.0	130	104	>40′	
C1	35.0	120	100	>1 hr.*	

* Normal blood pressure 6 days later

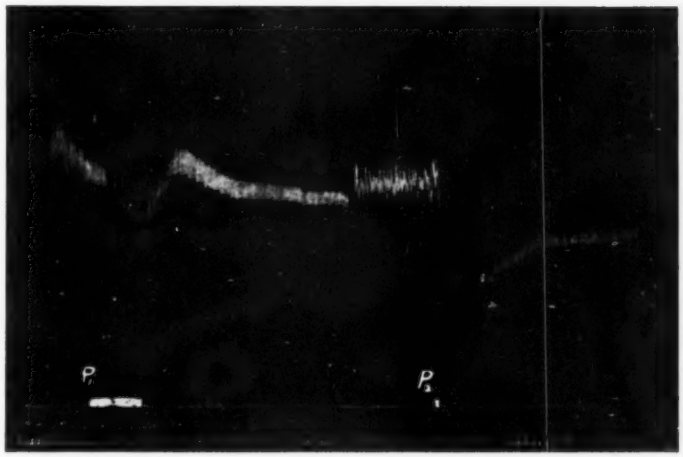

FIG. 3.—Effect of protamine on blood pressure. 5 mg./kg. protamine intravenously. P_1—injected in 4½ minutes; P_2—injected in 15 seconds, 70 minutes after P. Time marker —15-second intervals. Initial B.P. = 184 mm. Hg.

As illustrated in Fig. 1, a second injection of protamine in the dog is ineffective. This failure of response to a second dose lasts approximately four to six hours. In one animal a second injection after an interval of four hours produced a fall in blood pressure one-half that obtained with the first injection, and we have consistently obtained the original fall in blood pressure with a second injection twenty-four hours after the first.

As shown in Fig. 3, the rate of injection of protamine is a factor in developing shock in the dog: when 5 mg./kg. was injected over a period of 4½ minutes there was some irregularity in the blood pressure tracing but no typical fall. Seventy minutes later, the same dose of protamine was injected over a period of fifteen seconds, as in the other experiments, and the typical fall in blood pressure was obtained. Evidently the first slow injection of protamine was ineffective in causing the full fall in blood pressure and also failed to protect the animal against the effect of a second injection. It was of interest that the first injection had a marked effect on respiration. Marked dyspnoea was observed when one-third of the total amount of protamine had been injected. The fluctuations in blood pressure corresponded with the period of respiratory distress; this suggests that the respiratory effects of protamine can be dissociated from its depressor action.

The effect of pre-treatment of the dog on protamine shock

In order to investigate the nature of this action of protamine various agents were tested for their action on protamine shock (Table II). Mendel and Rudney (1944) have shown that protamine will inhibit the true cholinesterase at concentrations of an order which are physiologically significant. In two animals, the sensitivity to protamine was determined by the injection of 5 mg./kg. Twenty-four hours later, 0.02 mg./kg. of atropine was injected intravenously and five minutes later the injection of protamine was repeated. The same fall in blood pressure was obtained.

TABLE II

EFFECT OF PRE TREATMENT ON THE HYPOTENSIVE ACTION OF PROTAMINE IN THE DOG

Pentobarbitone anaesthesia; 5 mg. protamine per kg.

Dog	Pre-treatment	B.P. mm. Hg Before	After protamine	Recovery time in min.
S–11	—	112	20	8
,,	Atropine, 0.1 mg.	130	46	4.5
S–12	—	100	40	3.5
,,	Atropine, 0.2 mg.	96	14	4.5
C–10	Heparin 5 mg./kg. 20′ before	144	36	7
C–1		110	25	>40
C–30		154	69	>40
C–19	Heparin mixed with protamine *in vitro*	158	55	18
C–29		171	157	—
C–29	25 mg./kg. protamine 70′ later	142	36	>35
M9–5	Dicumarol 2 days previously	130	30	13
M9–9		170	56	15
M9–11		130	13	18
M–81	Eviscerated dog	116	52	8.5
M–06	Cord section ..	77	23	60 (fall occupied 2′)
M–08	,, ,, ..	124	46	1 (fall occupied 1′)
M–10	,, ,, ..	76	27	2 (fall occupied 2½′)
M–82	Benadryl, 100 mg. i.v.(*) (approx. 12 mg./kg.)	168	26	12
M–83	,, ,,	166	32	12
M–97	,, ,,	107	67	1 (fall occupied 1¼′)
M–98	,, ,, (*)	132	117	0.5 (fall occupied 2½′)
M–99	,, ,, (*)	175	97	25

* 10 mg. protamine per kg. in these experiments

Since the clinical use of protamine intravenously is likely to be chiefly for the purpose of neutralizing heparin, the effect of heparin on its depressor action was studied. Three dogs (C–10, C–1, C–30) were given 5 mg./kg. of heparin intravenously twenty minutes before the protamine; this assured an excess of heparin over the protamine in the circulation. As shown in Table II, heparin did not modify the effect of protamine on blood pressure; indeed, the shock appeared to be slightly more severe. In two other animals the protamine was mixed with the heparin in the syringe and the precipitate of heparin-protamine compound was injected; the toxic action of protamine still appeared. However, with dog C–29, when the compound had stood in the syringe for a longer time and the precipitate had formed larger masses, there was no fall in blood pressure; this was evidently a true neutralization of the toxic action of the protamine since the later injection of protamine itself caused the typical fall in blood pressure.

As shown below, intravenous injection of protamine causes the destruction of platelets. The liberation of thromboplastin from the platelets might cause shock similar to that reported by Mylon, Winternitz, Katzenstein, and de Suto-Nagy (1942). However, in three dogs given dicumarol two days previously, there was a typical fall in blood pressure after protamine. Removal of the abdominal viscera, including the liver, by the method of Markowitz (1937), did not alter the effect of protamine on blood pressure (M–81; Table II).

The effect of cord section on the action of protamine was also studied. In a series of dogs the spinal cord was severed between C2 and C3. Artificial respiration was started and the protamine injected in the usual way. A slow fall in blood pressure occurred after cord section. Injection of protamine resulted in a further superimposed fall in blood pressure, with partial recovery. The fall in blood pressure did not resemble that after the injection of protamine in the normal animal. Instead the development of the depressor action was much retarded and the fall in blood pressure was very slow. In animals similarly prepared but in which complete transection was not accomplished, protamine gave the typical fall in blood pressure. The results suggest that cord section affects the toxic action of protamine, particularly the rapid fall in blood pressure.

The effect of benadryl on the response to protamine was also studied (Table II). Irregular results were obtained which resembled those of Wells, Morris, Bull, and Dragstedt (1945) on the effect of benadryl in anaphylactic and histamine shock.

They found that, while complete inhibition did not result, benadryl sometimes largely neutralized the effect of the antigen. De Cuyper (1946) has reported that antergan counteracted the action of clupein on blood pressure, and it may reasonably be concluded that protamine shock is modified by antihistamine drugs.

Blood changes in protamine shock

The blood concentration of a number of factors was determined in protamine shock in the dog. Protamine concentration in the blood was determined by titrating its anticoagulant activity with heparin. At the neutralization point of these two anticoagulants, the clotting time is normal (Table III). Under the conditions used, 25 μg. of protamine

TABLE III

TITRATION OF PROTAMINE WITH HEPARIN

0.5 ml. of blood added to 25 μg. of protamine plus varying concentrations of heparin in 0.5 ml. of saline. ∞ means no coagulation

Protamine (μg.)	Heparin (μg.)	Clotting time (min.)
25	3.0	16
	4.0	13
	4.5	7
	5.0	8
	5.5	8
	6.0	22
100	10.0	∞
	15.0	∞
	20.0	26
	25.0	∞
	30.0	∞
0	0	6.5

was neutralized by 4.5 μg. of heparin. An interesting observation was made in connexion with these tests; the sensitivity of the test is greatly increased by diluting the blood with an equal volume of saline as described by Jaques, Charles, and Best. Control experiments showed that this dilution not only increased the normal clotting time and the clotting time with added heparin but also increased the clotting time with added protamine tenfold. Estimations of salmine present in the blood after intravenous injection of the protamine are reported in Table IV; 95 per cent of the injected protamine had disappeared within five minutes of the injection. The slow disappearance of the remainder is presumably due to the hydrolysis of protamine by plasma enzymes (Hagedorn, 1938).

The histamine and heparin contents of the blood were determined before and after the injection of

TABLE IV

CONCENTRATION OF PROTAMINE IN THE BLOOD AFTER INTRAVENOUS INJECTION

Injection—5 mg./kg. = 50 μg./ml. of blood

Time after injection of protamine	Clotting time min.	Heparin required to reduce the clotting time μg.	Protamine equivalent μg./ml.
Dog 8			
0	15.0	0	0
6 min.	15.0	0.3	3.0
3 hr.	8.0	0.1	1.0
21 hr.	6.5	0.04	0.4
Dog 10			
0	10	0	0
5 min.	10	0.3	3.0
32 min.	17	0.1	1.0
2 hr.	8	0.05	0.5
4 hr.	7	0.03	0.3

protamine (Table V). No increase in blood histamine was detected after the injection of protamine. The value for the normal blood histamine in Table V was abnormally high, but no increase was detected in the blood of the other animals of the series which showed normal values. Although these results were negative they do not allow the conclusion that the

TABLE V

BLOOD CHANGES IN PROTAMINE SHOCK

Dog M94. Protamine: 10 mg./kg.

Time from injection	B.P. mm. Hg	Blood histamine μg./ml.	R.B.C. × 10^3/cu.mm.	Platelets × 10^3/cu.mm.
—	160	2.0	5,150	95
—	160	2.0*	—	—
20″	140	—	6,850	35
90″	40	1.8	7,000	30
135″	18	1.8	—	—
190″	21	1.8	—	—
15′	69	1.7	6,500	15

* Blood sample + protamine, 0.1 mg./kg.

observed effect of protamine on blood pressure was not due to the liberation of histamine; 30 μg./kg. of histamine gave a fall in blood pressure in the anaesthetized dog equivalent to 10 mg./kg. of protamine so that the amounts of histamine liberated into the blood to give the fall in blood pressure observed may be too small to be determined by the methods used. In tests for histamine in the cat after atropine, an indication of an unstable depressor substance (not histamine) in the dog's plasma was

seen in one animal at the moment of lowest blood pressure.

Heparin was estimated by the method of Jaques, Monkhouse, and Stewart (1949). Heparin in blood can be determined by this method in concentrations above 0.8 μg./ml. (0.1 international units). No heparin was found in the blood after the injection of protamine. In view of the affinity of protamine for heparin, it would be of interest to study the mast cells in order to discover whether protamine had become localized there, but this was not done in the present study. The injection of protamine had little effect on the red cell count. The increase in count shown in Table V was not a constant accompaniment of the action of protamine.

The thrombocytopenic action of protamine

The most marked effect of protamine on the blood constituents was the marked fall in the white cell and platelet counts (Tables V and VI).

TABLE VI

EFFECT OF PROTAMINE ON CELL COUNTS

Protamine—5 mg./kg. in each experiment

Minutes after injection	B.P. mm. Hg	R.B.C. $\times 10^6$	W.B.C.	Platelets $\times 10^3$	Platelets agglutinated per cent
DOG—7.3 kg.					
0	160	6.526	7,600	408	0
1½	46	7.504	1,600	30	39
4	140	6.894	2,600	27	39
17	140	7.029	7,900	113	26
RABBIT—2.8 kg.					
0		5.662	3,100	349	11
2½		5.681	3,700	367	9
8		5.506	3,400	371	1

The fall in the platelet count was observed twenty seconds after the injection, before there was any fall in blood pressure. The maximum thrombocytopenia, with a count about 10 per cent of the normal, was generally reached in two to four minutes at the time of the maximum reduction in blood pressure. The recovery of the platelet count was quite rapid, but lagged behind the return to normal of the blood pressure. There was usually a significant rise in the count in fifteen minutes and the count was largely restored within an hour.

As shown in Tables VI and VII there appears to be some connexion between the extreme thrombocytopenia and the fall in blood pressure produced by protamine in the dog. In the rabbit with no fall in blood pressure, there was no change in the platelet count. In several rabbits a fall did occur, but it did not amount to more than 10 per cent. With repeated injections of protamine in the dog, it will be evident from Table VII that after the first injection, accompanied by a fall in blood pressure, there

TABLE VII

EFFECT OF REPEATED INJECTIONS OF PROTAMINE ON BLOOD PRESSURE AND CELL COUNTS IN THE DOG

Time	B.P. mm. Hg	R.B.C. $\times 10^3$/cu.mm.	W.B.C. per cu.mm.	Platelets $\times 10^3$/cu.mm.
10.20 a.m.	—	6,843	10,000	290
11.45	145	5,879	7,300	319
11.50	Protamine—5 mg./kg.			
11.53.30	30	—	—	—
11.56	100	5,188	3,700	47
12.30 p.m.	140	6,606	3,600	87
2.00	140	6,438	4,600	196
3.10	140	6,363	18,400	222
3.13.30	Protamine—5 mg./kg.			
3.14.50	150	7,446	5,100	172
3.19.10	144	7,220	14,900	173
3.59	144	7,213	17,100	223
9.27	144	6,620	27,000	261
9.36.10	Protamine—5 mg./kg.			
9.38.40	150	6,944	9,100	160

was a pronounced decrease in the platelet count to 15 per cent of the pre-injection value. However, on the second and third injection, the count was decreased by only 22 and 39 per cent. In our studies on platelets we have found in general that a fall in the platelet count of 20 or 30 per cent is caused by many substances and is a general type of reaction, whereas a fall in the platelet count of 80–90 per cent is a more specific effect. Hence the thrombocytopenia after the second injection may be not only quantitatively but also qualitatively different from the first.

In addition to the thrombocytopenia, the intravenous injection of protamine also caused a leucopenia in the dog (Tables VI and VII), but, as with the platelets, the white cells were not affected in the rabbit. In contrast to the changes observed in the platelet count, with repeated injections of protamine in the dog there was a greater decrease in the leucocyte count on the second and third injections.

In order to determine whether it affected the platelets and white cells directly, protamine was mixed with blood outside the body and counts performed on the mixture. Since the properties of the platelets are markedly affected by anticoagulants, the silicone technique described by Jaques, Fidlar, Feldsted, and Macdonald (1946) was used. By this technique it is possible to keep blood for over

30 minutes without a decrease in the platelet count. The needles and syringes for drawing the blood were coated with this material. 5 ml. of blood were drawn and 1 ml. added to 0.1 ml. of protamine solution (50 μg.) in a silicone-coated beaker; 1 ml. of blood was also added to 0.1 ml. of saline in a similar beaker to act as a control. As shown in Table VIII, there was relatively little change in the platelet count in the control beaker in eleven minutes. The decrease in count observed was probably due to sedimentation causing difficulties in

TABLE VIII

EFFECT OF PROTAMINE ON BLOOD IN SILICONE

Clotting time of blood in silicone: >2 hr.

1 ml. of sample blood + time		R.B.C. × 10^6/cu.mm.	W.B.C. cu.mm.	Platelets cu.mm.
0.1 ml. saline	1′	5.794	14,500	288,000
	11′	7.638	18,000	264,000
0.1 ml. protamine (50 μg.)	2′	7.075	15,900	314,000
	12′	7.075	18,000	40,000

obtaining a representative sample, since the red cell and leucocyte counts showed a corresponding increase. However, in the sample with added protamine, there was a very marked decrease in the platelet count of 87 per cent, although there was no decrease in the white cell count. It is evident that protamine affected the platelets directly whereas it had no direct effect on the leucocytes. The decrease in the platelet count may have been due either to lysis of the platelets or to agglutination of the platelets with settling out of the platelet masses. This is possible both *in vivo* and in the silicone beaker, although the very marked decrease in the platelet count in the latter with no evidence of agglutinated platelets suggests that lysis has occurred. Actually the two phenomena are not necessarily distinct, since platelet agglutination is frequently followed by " viscous metamorphosis " and eventual solution of the platelets. Hence, while agglutination could be the chief factor *in vivo* and is illustrated in Table VI, this is probably succeeded by lysis in the silicone beaker.

The fact that the white cell count is not affected by protamine in silicone suggests that the fall in the white cell count which occurs *in vivo* is not a direct action of the protamine. Such a decrease in the white cell count occurs, however, whenever there is agglutination of platelets, as the white cells stick to the platelet clumps which are blocked in the capillaries. This suggests that agglutination of the platelets is the chief phenomenon on injecting protamine *in vivo*, and that the leucopenia is secondary to this.

Since an effect of protamine on the platelets could be detected *ex vivo* by means of the silicone technique, a direct comparison was made of the action of protamine on platelets *in vivo* and *ex vivo*. A sample of blood was taken and added to protamine and saline in silicone as before. Samples for platelet counts were taken after 10 sec., 2, 5, and 10 min. A corresponding quantity of protamine (5 mg./kg.) was injected intravenously and samples for platelet counts taken at the same time intervals. The experiment was repeated three hours and ten hours later. The results are shown in Table IX. It will

TABLE IX

COMPARISON OF THE EFFECTS OF PROTAMINE ON PLATELETS *in vivo* AND *ex vivo*

Protamine = 5 mg./kg. = 0.05 mg./ml. of blood

Time	Platelets × 10^3/cu.mm.		
	Ex vivo	Saline control	*In vivo*
0	—	—	120
10″	90	110	80
2′	70	115	25
5	50	110	20
10′	—	—	20
120′ (0)			90
10″	65	105	65
2′	60	100	30
5′	45	90	30
10′	50	—	65
10″	70	65	—
2′	40	70	65
5′	40	60	60
10′	45	—	25
60′			20

be evident that the thrombocytopenia observed after the injection of protamine also occurred at the same time *ex vivo*, although, since the fall was not as great, part of the thrombocytopenia after the injection of protamine is due to filtering out of platelet clumps in the capillaries. A difficulty in interpretation in this experiment, compared to that of Table VII, was caused by the fact that the initial platelet count of this animal was low and the recovery of the count much poorer, so that, while the decrease in the count was less after the second and third injection, the thrombocytopenia was just as severe.

The effect of glycogen on the toxicity of protamine

From the above it is evident that the agglutination of platelets and accompanying white cells is inde-

pendent of the effect of protamine on blood pressure, at least as far as the moderate thrombocytopenia occurring on a second and third injection of protamine. However, it is possible that the extreme fall in the platelet count after the first injection is related to the fall in blood pressure at this time. As mentioned previously, the fall in the platelet count actually begins before the fall in blood pressure. However, this does not give us any indication whether the fall in blood pressure is the cause or the result of the platelet agglutination associated with the marked fall in the platelet count. Rocha e Silva, Grana, and Porto (1945) found that the intravenous injection of glycogen will produce a temporary disappearance of the platelets from the blood, and they have used this as a method of studying the contribution of the platelets to various types of anaphylactoid shock. Four dogs were therefore given glycogen (1 g./kg.) intravenously before the injection of protamine (Table X). A complete

TABLE X

EFFECT OF THE INJECTION OF GLYCOGEN ON THE ACTION OF PROTAMINE

Expt.	Platelet count $\times 10^3$/cu.mm.			B.P., mm./Hg		
	Before glycogen	Before protamine	After protamine	Before protamine	After protamine	Recovery time
M–64	402	119	114	107	28	6′
M–00	75	9.2	4.3	98	82	20″
M–02	110	68	3.8	102	28	12′
M–11	108	9	5	159	143	15″
M–11*	—	80	8	116	61	20″

* 2 hours after first injection

disappearance of the platelets from the circulation after injecting glycogen was not found.

In the two experiments where a thrombocytopenia was produced (count less than 10,000/cu.mm.), the effect of protamine on the blood pressure was largely abolished. These were the only cases, in a large series of dogs, in which the intravenous injection of 10 mg./kg. of protamine in ten seconds did not cause the typical fall in blood pressure. This suggests that the thrombocytopenia produced by protamine is an important factor in its hypotensive action.

Discussion

It is evident from the work of Vartiainen and Marble, Shelley, Hodgkins, and Visscher, and others, that protamine is toxic to rats, guinea-pigs, and mice with an intravenous LD50 about 100 mg./kg. Smaller doses had relatively little effect, and Shelley, Hodgkins, and Visscher explained the effect as due to embolic vascular phenomena since protamine in these concentrations on addition to whole blood produced a thready precipitate and a gross agglutination of the blood corpuscles, related to the precipitation of fibrinogen by protamine, observed by Mylon, Winternitz, and de Suto-Nagy (1942). It seems probable that this action of protamine is responsible for the toxicity of protamine in the guinea-pig and other species, but it is unlikely that this is responsible for the toxicity in the dog. Mylon, Winternitz, and de Suto-Nagy state that it required 1.5 mg. of protamine per ml. of plasma to precipitate 80 per cent of the total fibrinogen and 0.5 mg. per ml. to affect the fibrinogen as judged by lengthening of the clotting time. A concentration of 0.05–0.10 mg. per ml., equivalent to the doses injected (5–10 mg. per kg.), fails to give a precipitate with oxalated plasma.

Not only is the action of protamine in the dog distinguished by its much lower dose level, but it is also differentiated by the nature of the toxic reaction. The chief effect in the dog is a temporary fall in blood pressure. This reaction depends markedly on the nature of the injection. It is produced only by a relatively rapid intravenous injection of protamine, and an effective fall in blood pressure provokes a tachyphylaxis lasting for some hours.

An attempt has been made to elucidate the nature of the hypotensive action of protamine. Fundamentally, the effect is due to vasodilatation of arterioles. This is shown by the simple but consistent observation that a venous sample taken when the blood pressure is falling is bright red and arterial in colour. Also, electrocardiographic tracings of a dog showed no change during the injection of salmine. Since the effect is still observed in the eviscerated animal, the liver and splanchnic area do not appear to take a great part in the action of protamine, but rather the muscular vessels are chiefly involved, as indicated by Thompson. The action of protamine in increasing capillary permeability through displacement of platelets and serum proteins discovered by Danielli (1940) is not a factor in the present experiments since haematocrit determinations did not show any immediate change. A very slight haemoconcentration occurred five minutes after the injection.

The vasodilatation and resulting fall in blood pressure after protamine might be due (1) to a direct effect on smooth muscle, or (2) to the liberation of histamine or other similar agents, or (3) to a reflex due to stimulation either of chemoreceptors, etc., in

the circulation or of the vasomotor centres. As indicated above, protamine possesses no histamine-like action on guinea-pig smooth muscle, and likewise it was not found to have any significant effect on the isolated dog ileum. This suggests that the action is not a direct effect on smooth muscle. The results on the spinal animals indicate that the action of protamine is not a simple one, but that, while the sharp initial fall in blood pressure may be due to a reflex vasodilatation, this is accompanied by a more slowly acting hypotension, suggestive of the release of pharmacologically active substances.

Since several protein products, simple proteins, and organic bases produce anaphylactoid shock in the dog by causing the liberation of histamine, one would expect a basic protein like protamine to have this action. Although we failed to detect histamine in the blood during protamine shock in the dog, the shock is relatively mild, and hence the amounts of histamine liberated may be quite small. The fact that the shock, on occasion, was modified by the previous administration of benadryl suggests that histamine release does occur and is a factor in the shock produced by protamine.

It is evident from the results obtained that associated with the effect of protamine on blood pressure in the dog is a marked thrombocytopenic effect. The very great fall in platelet count to 15 per cent of normal occurred only with the fall in blood pressure. Further, in contrast to leucocytes, the platelets were clumped and lysed by protamine directly. The production of a thrombocytopenia by the injection of glycogen prevented the depressor action of protamine, whereas failure to produce a thrombocytopenia with the same dose of glycogen resulted in failure to neutralize the action of protamine. This suggests that the action of protamine on the platelets plays an important role in the hypotensive effect of this substance. Possible mechanisms whereby platelet changes may cause a fall in blood pressure are suggested by the reports of Mylon, Winternitz, Katzenstein, and de Suto-Nagy (1942) and Rocha e Silva and Texeira (1946). Mylon *et al.* reported that thromboplastic shock could be prevented by cord section, which suggests that platelet changes could be responsible for that fraction of the depressor action of protamine prevented by cord section. On the other hand, Rocha e Silva and Texeira have suggested that in peptone shock platelet agglutination is responsible for the liberation of histamine. However, it should be pointed out that the thrombocytopenia may be a phenomenon accompanying the fall in blood pressure, rather than the cause of the hypotension, as the only experiments which provide evidence of a causal relationship are the few experiments in which glycogen was injected.

Rocha e Silva and Texeira (1946) have postulated that in peptone shock platelet disintegration leads to activation of plasma protease or fibrinolysin and that this causes the liberation of histamine. Since protamine can be used to demonstrate the presence of active fibrinolysin in blood, owing to its inhibition of the natural protease inhibitor (Scroggie, Jaques, and Rocha e Silva, 1947) this action of protamine might be responsible for its toxicity. Blood samples were taken from several animals and inspected for fibrinolysis, both by examining the blood directly and also by conducting the tests of Rocha e Silva and Texeira (1946) and MacFarlane (1946). No marked increase in fibrinolysis was observed after the injection of protamine. Activation of fibrinolysin may be a factor in the toxicity of protamine, but no evidence of this was seen in the present experiments, while in the experiments of Rocha e Silva and Texeira (1946), and of Mylon, Winternitz, and de Suto-Nagy (1942), the concentration of protamine used for fibrinolysis was much higher than the dosages used above.

It was observed that the effect of protamine on the platelets resulted in poor haemostasis as the animal bled freely from venipuncture wounds after the injection. This did not appear to be associated solely with the thrombocytopenia, as it appeared to persist even after the platelet count had risen again. Minor changes in the prothrombin time, referable to the effect on platelets and fibrinogen, were observed after protamine.

In addition to the effects on blood pressure in the dog, the protamines also affect respiration and the gastro-intestinal tract. These effects were minimal in the present study, and a separation of the respiratory and depressor effects of salmine was indicated with repeated injections. Thompson (1900) observed much greater toxicity when using clupein. The thymus histone studied previously gave results more closely resembling those of Thompson both in the effect on respiration and the duration of hypotension. In this connexion, it is of interest that the results reported by Jaques, Charles, and Best show that a fall in blood pressure due to the thymus histone did not produce refractoriness to salmine. Hence, while the various protamines and related basic proteins give similar pharmacological reactions, they are by no means interchangeable in this respect. This suggests that it is advisable to refer to them by their specific names—salmine, clupein, scombrin, etc., rather than by the generic name of protamine. It is interesting, in view of the effect of cord section on the depressor action, to note that the gastro-

intestinal symptoms reported by Jaques, Charles, and Best have been reported only in the unanaesthetized animal.

Since many of the symptoms resulting from the injection of protamine in the dog resemble a mild anaphylactic shock, this may be a heterophil anaphylaxis, like the sensitivity to *Ascaris suis* reported by Rocha e Silva, Grana, and Porto (1945). Skin tests and precipitin tests for protamine in dogs were conducted but were negative. Likewise, attempts to develop antibodies in guinea-pigs were negative. This agrees with reports in the literature that protamine is non-antigenic.

It is evident from the above that among the common experimental animals protamine is most toxic to the dog. It appears that protamine has been given as an intravenous injection clinically on occasion, and up to the present no alarming symptoms have been reported. The effect of protamine on human platelets has been tested by us, using the same technique as in Table VIII. There was a definite decrease in the platelet count (150,000 to 85,000 in ten minutes), but it was much less than the corresponding decrease in the dog. It is therefore unlikely that the toxicity of protamine will be of significance in its clinical use. The finding that no toxic effects resulted in the dog if the protamine were injected slowly suggests an additional reasonable precaution.

Summary

1. The protamine, salmine, when rapidly injected intravenously was toxic to the dog in doses of 5 mg. per kg. At this dosage it showed little toxicity on intravenous injection in cats, rabbits, and guinea-pigs.

2. The intravenous injection of salmine in the dog caused a pronounced fall of blood pressure with rapid recovery and a slight apnoea. A second injection within six hours was without effect. A slow rate of injection did not cause a fall in blood pressure nor produce tachyphylaxis to a subsequent rapid injection. This effect of salmine on blood pressure was not changed by previous administration of atropine or of heparin, or by removal of the abdominal viscera. It was modified by the previous administration of benadryl, and by section of the spinal cord, and prevented by the previous intravenous injection of glycogen, when accompanied by thrombocytopenia. The fall in blood pressure was the result of extensive vasodilatation in the arterioles of muscles.

3. No increase in the amount of blood histamine or blood heparin could be detected after the injection of salmine. A definite transient thrombocytopenia and leucopenia occurred. Severe thrombocytopenia was associated with the fall in blood pressure, but leucopenia and a moderate fall in the platelet count occurred after the injection of the protamine in the absence of the fall in blood pressure.

4. Salmine added to blood in silicone-treated vessels caused thrombocytopenia but not leucopenia.

The author is greatly indebted to Professor C. H. Best and Dr. E. Fidlar, of the University of Toronto, for their interest in the problem. An active part in certain phases of the investigation was taken by Miss Mary Stewart, Miss A. E. Scroggie, Miss J. G. Cooper, and Miss Erica Lepp, and portions of this study were presented by the first three students as a part of the requirement for the honours degree in physiology and biochemistry at the University of Toronto. He is indebted to Mr. F. C. Monkhouse for the blood heparin determinations and to Dr. A. M. Fisher, of the Connaught Medical Research Laboratories, for the protamine. The investigation was assisted by grants from the John and Mary R. Markle Foundation to the University of Toronto and from the National Research Council of Canada to the University of Saskatchewan.

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A CONTRIBUTION TO THE PHYSIOLOGY AND PHARMACOLOGY OF *ASCARIS LUMBRICOIDES* FROM THE PIG

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(Received December 17, 1948)

The observations reported in this paper arose indirectly out of an attempt to devise a logical and satisfactory method for the detection of anthelminthic potency *in vitro*. In the past, and mainly on the authority of Trendelenberg (1916), *in vitro* tests for anthelminthics have usually been carried out on earthworm muscle (e.g., by Lautenschläger, 1921; Oelkers and Rathje, 1941; von Oettingen, 1929; Rosenmund and Schapiro, 1934), some workers supplementing this by leech preparations. Since, however, the Nematoda show no trace of the metameric segmentation which characterizes the Annelida and, indeed, differ from them in almost every respect (Chitwood and Chitwood, 1937–8; Lapage, 1937), this seems an entirely unjustifiable procedure unless it can first be demonstrated that members of the two phyla do in fact react in substantially the same manner to a diversity of physiological and pharmacological agents. But Lamson and Ward (1936) carried out comparative tests of no less than 121 different chemical substances on earthworms and on pig *Ascaris*; they could find no correlation between the respective responses, and they condemned the use of earthworm material in anthelminthic studies as "irrational."

In our own work we have been concerned only with nematodes, and the use of the term "anthelminthic" in the present paper is accordingly restricted to substances which are anthelminthically active towards nematodes; the observations have been made exclusively with *Ascaris lumbricoides* obtained from the pig. Our earlier *in vitro* method (Baldwin, 1943) made use of tied-off, sausage-like pieces of *Ascaris*, these "sausages" being surrounded by the cuticle which is such a prominent and important feature of the Nematoda. Two kinds of "sausages" were used—viz., "anterior" preparations each comprising a terminal anterior portion about 2 cm. in length, and "intermediate" preparations taken from the region immediately in front of the genital pore and about 2.5 cm. in length.

A method based on the use of intact specimens of pig *Ascaris* had previously been introduced and extensively used by Lamson and his colleagues (1935, 1936). We, however, doubted the reliability of this procedure because of the well-known resistance, first recorded by von Schroeder (1885), of intact *Ascaris* to a number of anthelminthics, including even nicotine and santonin. In our anterior "sausage" preparations, on the other hand, santonin produces complete paralysis within a few minutes at concentrations of 10^{-5}; this effect seems to be due to a specific action of santonin upon the central nervous system, the "nerve ring," which is situated very close to the anterior extremity (Rauther, 1925).

With these "sausages" a strong correlation was established between positive reactions in the tests and acknowledged anthelminthic potency in the substances tested. At the same time, however, the "sausage" preparations showed not the slightest response to a wide range of pharmacologically active compounds such as acetylcholine and adrenaline and drugs which potentiate, imitate, or antagonize them. It seemed desirable to discover why these drugs should be inert. Two possibilities suggested themselves: (*a*) these drugs might fail to penetrate the cuticle, which is known to show highly selective permeability (Trim, 1949), or (*b*) nematode tissues might be insensitive to these substances. In either case we should have powerful arguments against the use of annelid preparations for anthelminthic testing, since neither the earthworm nor the leech possesses anything comparable

with the nematode cuticle, while both are notoriously sensitive to many compounds which possess no known anthelminthic activity.

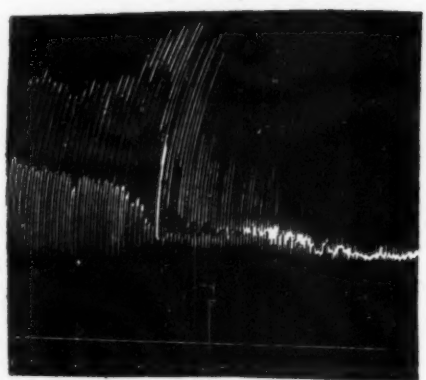
Our first problem consisted in devising a nematode preparation in which the substances to be investigated could be directly applied to the muscle. The preparation devised, and a saline medium suitable for its maintenance, have already been described (Baldwin and Moyle, 1947).

The present paper reports the results of a study of the reactions of such "exposed" preparations towards a number of compounds of physiological and pharmacological interest, most of which are devoid of action upon the earlier "sausage" preparations (Baldwin, 1943). The observations are offered as a contribution to the at present scanty knowledge of the physiology and pharmacology of the Nematoda. They provide at the same time fresh factual evidence of the complete unsuitability of annelid material for tests of anthelminthic activity.

Results

1. *Perfusion experiments.*—Before we succeeded in obtaining active preparations of exposed *Ascaris* muscle we carried out a number of experiments in which short pieces of the worm were taken from the "intermediate" region—i.e., immediately in front of the genital pore. Fine cannulae were tied between the gut and the muscle masses of these preparations, which were then perfused with physiological saline, with and without the addition of various drugs. Perfusion was carried out with a mechanically operated syringe at a rate of about 6 ml. per hour and the movements executed by the muscles were kymographically recorded in the same manner and with the same apparatus as was used with the original "sausages" (Baldwin, 1943). Thymol and hexylresorcinol (Fig. 1*a*) were tested at concentrations of 5×10^{-4}, and, as was to be anticipated from their action upon the "sausage" preparations, they produced rapid paralysis and subsequent contracture of the muscle. Santonin (Fig. 1*b*) at 2×10^{-4} led to a near-paralysis, while acetylcholine (10^{-6}) now produced marked stimulation of the muscle. Pilocarpine and adrenaline (10^{-3}) were quite inert, and *p*-benzylphenyl carbamate, which produces paralysis in 20–30 min. when applied at 2×10^{-4} to the "sausages," was quite without action when applied by perfusion. These results seemed to justify a renewal of our efforts to obtain active preparations of the exposed muscle, especially since santonin, which paralysed our perfused preparations, is known to produce violent stimulation of denervated earthworm muscle (Trendelenberg, 1916). Furthermore, we now had evidence of sensitivity of the *Ascaris* tissue towards acetylcholine. At about this time we began to get more promising results with exposed preparations, and the perfusion technique, which proved tedious and not a little difficult, was abandoned soon after the pilot experiments just mentioned had been completed.

(*a*)



(*b*)

FIG. 1.—Perfused preparation of *Ascaris*. (*a*) 5×10^{-4} hexylresorcinol at signal; (*b*) 2×10^{-4} santonin at signal.

2. *Experiments with exposed preparations.*—The technique has already been described (Baldwin and Moyle, 1947). Each preparation consists of a ribbon-like strip of dorsal musculature about 2.5 cm. in length taken from the "intermediate" region. The cuticle is still present on the external face of the strip, but the other side is exposed directly to the bathing medium. These preparations are sometimes exceedingly sensitive to mechanical stimulation so that, when the bathing medium is changed, the greatest care must be taken to avoid spoiling the tracings.

The Table summarizes the effects of a number of drugs when applied to the exposed preparations: their effects upon the "sausages" (Baldwin, 1943) are given side by side for comparison.

TABLE

THE CONCENTRATIONS ARE, WITH INERT COMPOUNDS, THE HIGHEST AT WHICH THEY WERE TESTED AND, WITH ACTIVE COMPOUNDS, THE LOWEST AT WHICH ACTIVITY WAS STILL DETECTABLE

s and *p* indicate that stimulant or paralysant effects respectively were observed within 30 min. at the concentrations indicated. ± indicates a weak or dubious response and — that no effect could be detected

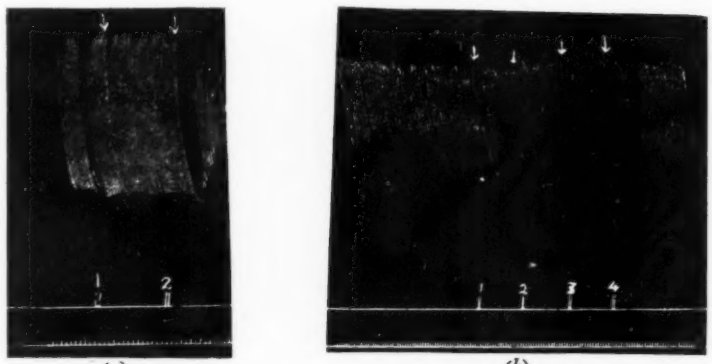
Compound	Exposed preparation		"Sausage" preparation	
	Concentration	Effect	Concentration	Effect
A				
Acetarsone	10^{-4}	—	2×10^{-3}	—
Acetylcholine	10^{-6}	*s*	2×10^{-4}	—
Adrenaline	10^{-4}	—	2×10^{-4}	—
Arcain	10^{-4}	—	10^{-3}	—
Atropine	10^{-3}	—	10^{-3}	—
Caffeine	10^{-3}	—	10^{-3}	—
Chloral hydrate	10^{-3}	±	10^{-3}	—
Choline	10^{-4}	*s*	10^{-3}	—
Cocaine	10^{-4}	*p*	10^{-3}	—
Ephedrine	10^{-4}	—	10^{-3}	—
Histamine	10^{-4}	—	5×10^{-4}	—
Morphine	10^{-4}	—	10^{-3}	—
Pelletierine	10^{-4}	*s*	10^{-3}	—
Phenothiazine	sat. soln.	—	10^{-3}	—
Phenylurea	10^{-3}	—	10^{-3}	—
Pilocarpine	10^{-4}	—	10^{-3}	—
*Pseudo*aconitine	10^{-3}	—	10^{-3}	—
Quinine	10^{-3}	±	10^{-3}	—
Strychnine	10^{-3}	—	10^{-3}	—
Sulphanilamide	10^{-4}	—	10^{-2}	—
Sulphapyridine	10^{-4}	±	10^{-3}	—
Sulphathiazole	10^{-4}	—	5×10^{-5}	—
Tyramine	10^{-4}	—	10^{-3}	—
B				
Arecoline	10^{-4}	*p*	10^{-3}	±
,,	10^{-3}	*s*	10^{-3}	±
Coumarine	10^{-3}	*p*	10^{-3}	—
Santonin	10^{-5}	*p*	2×10^{-4}	±
C				
p-Benzylphenyl-carbamate	10^{-4}	*p*	2×10^{-4}	*p*
Hexylresorcinol	10^{-4}	*s*	10^{-4}	*p*
β-Naphthol	10^{-4}	*p*	2×10^{-4}	*p*
Nicotine	10^{-7}	*s*	5×10^{-4}	*p*

Within Group A (drugs devoid of action upon "sausages") there are four compounds that produce definite effects when applied directly to the muscle, indicating that their failure to act upon the "sausages" must be attributed to inability to penetrate the cuticular layer. In Group B (drugs which paralyse "anterior sausages" but have little action upon "intermediate sausages") all three compounds tested showed considerably greater activity and acted at lower concentrations upon the "exposed" than upon the "sausage" preparations, again indicating slowness or failure of penetration of the cuticle. In Group C (compounds which act both upon "anterior" and "intermediate sausages") the four compounds examined again showed activity, but, whereas the two phenolic substances and the phenol carbamate showed about the same activity whether applied directly or by way of the cuticle, which they may therefore be presumed to penetrate rapidly, nicotine was far more active when directly applied. This presumably indicates that nicotine can pass only relatively slowly through the cuticle.

These preliminary experiments showed that the resistance of the "sausages" to pharmacological agents is at least sometimes due to the protective action of the cuticle. The fact nevertheless remains that *Ascaris* muscle, even when directly exposed, fails to react to such compounds as adrenaline, histamine, pilocarpine, strychnine, and many others.

This made it desirable to study more closely its general pharmacological behaviour. We accordingly carried out further experiments on the influence of a number of substances including adrenaline, acetylcholine, and drugs which simulate, potentiate, or antagonize their usual effects.

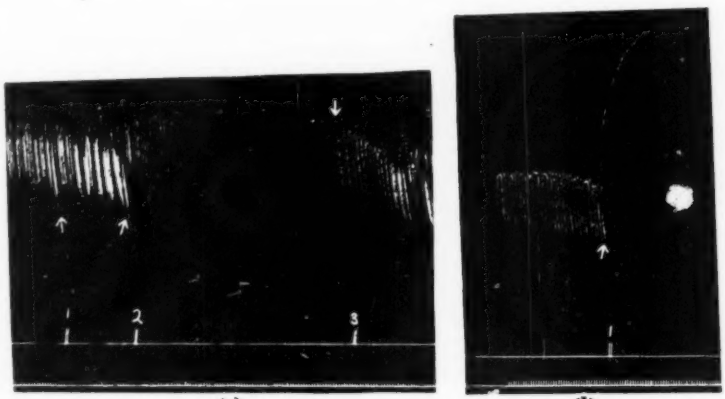
Adrenaline, ephedrine, ergotoxine.—Adrenaline was without detectable influence at concentrations up to 10^{-4}, whether applied alone or in the presence of ephedrine (10^{-4}), which itself was likewise inert. Ergotoxine, which antagonizes the augmentor effects of adrenaline, was also inert at 10^{-4}, whether applied before or after the preparations had been treated with adrenaline.



(*a*) (*b*)

FIG. 2.—"Exposed" preparation of *Ascaris*. (*a*) 10^{-5} eserine at 1; 10^{-6} acetylcholine at 2. (*b*) Acetylcholine, 10^{-6} at 1, 10^{-5} at 3; wash at 2 and 4.

Acetylcholine, eserine, choline.—At concentrations below 10^{-6} acetylcholine is inert, even after previous treatment with eserine (10^{-5}). Eserine alone (10^{-5}) is also without action (Fig. 2*a*). At 10^{-6} acetylcholine (Fig. 2*b*) produces a typical stimulation marked by a sharp rise of tone and corresponding diminution of amplitude. Higher concentrations gave only an exaggeration of this response; at 10^{-5}—10^{-4} the rise of tone is so great that relaxation often fails altogether. These effects are imitated by choline, which, at 10^{-4}, produces responses that are qualitatively and quantitatively similar to those of acetylcholine at 10^{-6}—10^{-5}. The effects of acetylcholine, which are reversed by washing, are quite uninfluenced by previous treatment of the preparation with eserine and do not fall off with time. It is difficult therefore to escape the conclusion that cholinesterases are totally absent from our preparations.



(*a*) (*b*)

FIG. 3.—"Exposed" preparation of *Ascaris*. (*a*) Nicotine, 10^{-8} at 1, 10^{-7} at 2; wash at 3. (*b*) 10^{-3} nicotine at signal.

Nicotinic effects: nicotine, tubocurarine, strychnine, cocaine.—Nicotine at 10^{-7} (Fig. 3*a*) exactly reproduces the effects of acetylcholine at 10^{-6}—10^{-5}. Higher concentrations (10^{-5}) evoke responses of the same qualitative kind but of greater intensity; even at 10^{-3} there is no indication of any reversal of the stimulatory effects (Fig. 3*b*). Thus nicotine does not have the paralysant action which it so often displays at high concentrations in other tissues.

The effects of nicotine at 10^{-7} are abolished by tubocurarine (Fig. 4*a*) at 10^{-4}, a concentration which, in the absence of nicotine, leads to complete paralysis after a short time. Tubocurarine at 10^{-4} also abolishes the effect of acetylcholine at 10^{-5} (Fig. 4*b*). Strychnine (10^{-3}) resembles tubocurarine in abolishing the action of acetylcholine (10^{-5}) (Fig. 4*c*) but differs from it in having no obvious or reproducible effect when applied by itself (10^{-3}). Cocaine at 10^{-4}, like tubocurarine, also leads to complete paralysis, presumably by narcosis, but full activity can be restored by nicotine at 10^{-7}. At higher concentrations (10^{-3}), however, the action of cocaine is no longer abolished by nicotine (10^{-5}).

These results show clearly that nicotine can imitate the effects of acetylcholine upon the *Ascaris* tissues. The influence of nicotine is also imitated by Ba^{++}, which at 10^{-4} has effects exactly comparable with those of nicotine at 10^{-7} or acetylcholine at 10^{-6}—10^{-5} (Fig. 4*d*). In addition, arecoline, which is reputed to have nicotine-like effects in high concentrations (Feldberg and Vartiainen,

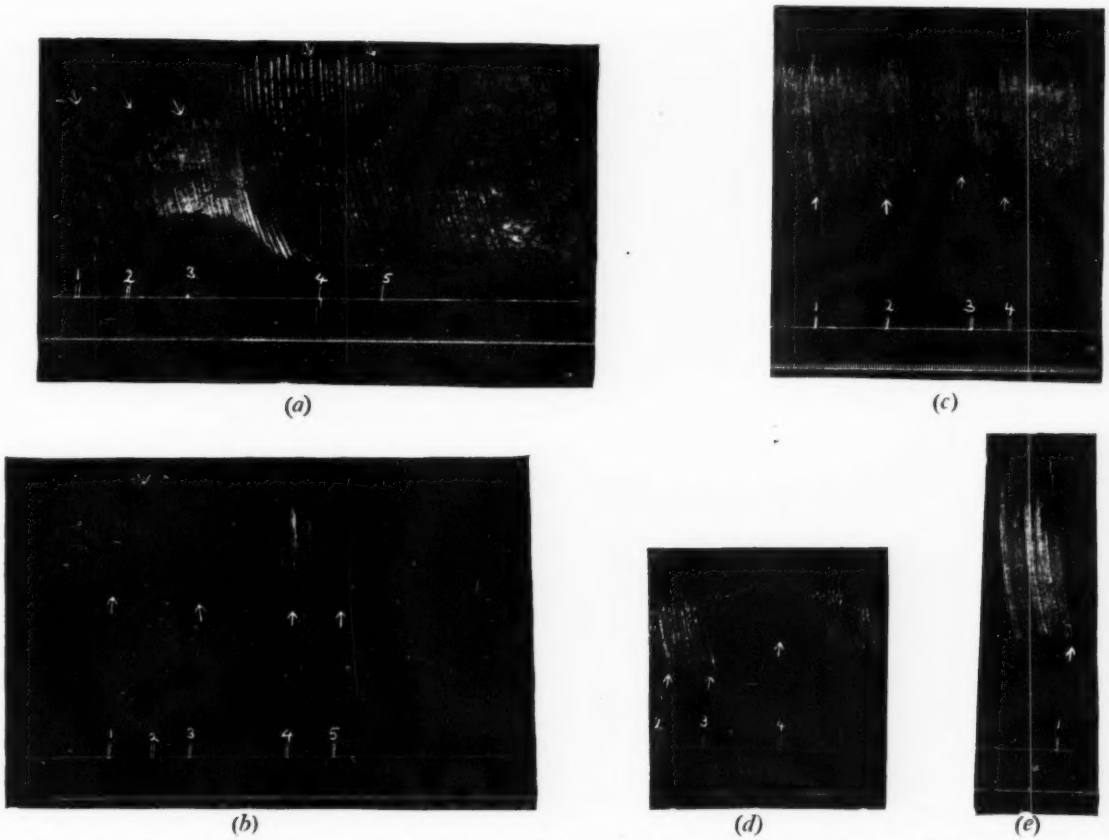


FIG. 4.—"Exposed" preparation of *Ascaris*. (*a*) Tubocurarine-nicotine antagonism: tubocurarine, 10^{-6} at 1, 10^{-5} at 2, 10^{-4} at 3; 10^{-4} tubocurarine + 10^{-7} nicotine at 4; 10^{-4} tubocurarine + 2×10^{-7} nicotine at 5. (*b*) Tubocurarine-acetylcholine antagonism: 10^{-5} acetylcholine at 1; wash at 2 and 3; 10^{-4} tubocurarine at 4; 10^{-4} tubocurarine + 10^{-5} acetylcholine at 5. (*c*) Effect of strychnine: 10^{-4} at 1, 10^{-3} at 2; 10^{-3} strychnine + 10^{-5} acetylcholine at 3; wash at 4. (*d*) Effect of barium: $BaCl_2$, 10^{-5} at 2, 10^{-4} at 3; wash at 4. (*e*) Effect of arecoline (high concentration): 10^{-3} at signal.

1935), produces at 10^{-3} a stimulant effect quantitatively comparable with that of nicotine at 10^{-6}—10^{-5} or acetylcholine at 10^{-5}—10^{-4} (Fig. 4*e*).

While nicotine faithfully imitates the action of acetylcholine at low or moderate concentrations it cannot be concluded that acetylcholine has a typical "nicotine-action" upon *Ascaris* tissue, since, according to the classical definition of Dale (1914), such effects are characteristically abolished by high concentrations of nicotine.

Muscarinic effects: pilocarpine, atropine, 2,268 F.—The effects of acetylcholine are not influenced in any way by atropine in concentrations as high as 10^{-4}, nor is atropine alone active at this concentration (Fig. 5*a*). This appears to show that the action of acetylcholine in this tissue does not include any "muscarine-action" (cf. Dale, 1914). Pilocarpine, which ordinarily reproduces the muscarinic effects of acetylcholine and of muscarine itself, is quite inert at 10^{-4}. Arecoline, however, which is reputed to simulate the muscarinic effects of acetylcholine when applied at low concentrations, produces paralysis at 10^{-4} (Fig. 5*b*), though it is inert at lower concentrations and has at 10^{-3} a stimulant action, resembling that of acetylcholine and nicotine (Fig. 4*e*); this is the only indication so far of any muscarine-like response by the *Ascaris* tissue.

Muscarine itself was not procurable and we were therefore obliged to have recourse to 2,268 F (ethylal-trimethylammonium-propanediol), a compound which is reputed to imitate faithfully the pharmacological effects of natural muscarine (Fourneau, Bovet, Bovet, and Montezin, 1944). This substance proved inert at 10^{-6} but at 10^{-5} produced an effect closely resembling that of nicotine at 10^{-7} or acetylcholine at 10^{-6}—10^{-5} (Fig. 5*c*). Its effect was not antagonized by atropine at 10^{-4} (Fig. 5*d*).

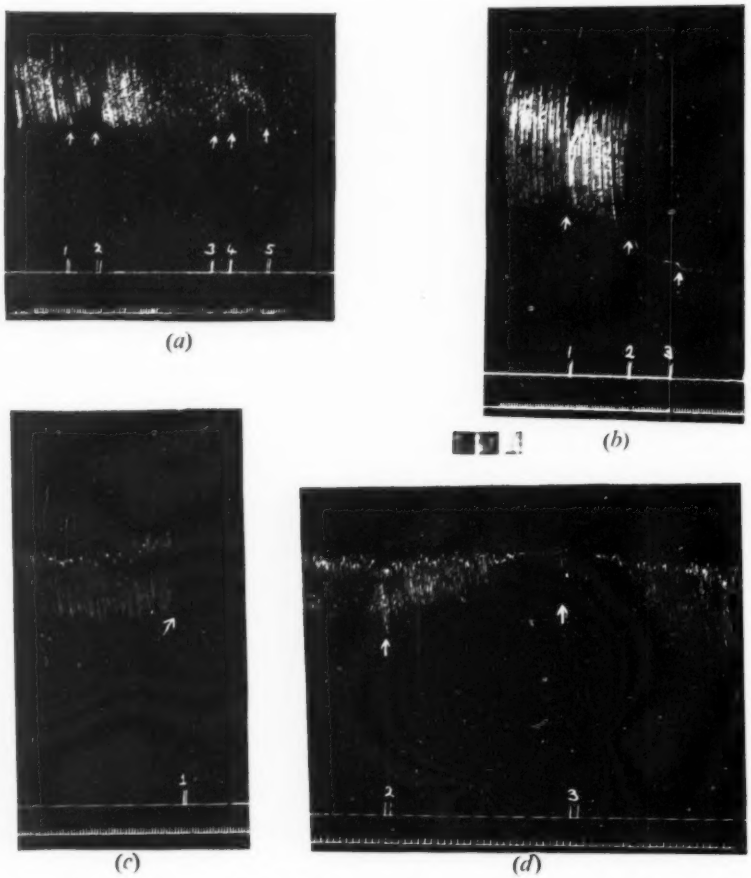


FIG. 5.—"Exposed" preparation of *Ascaris*. (*a*) Effect of atropine: 10^{-6} acetylcholine at 1; wash at 2; 10^{-4} atropine at 3; 10^{-4} atropine + 10^{-6} acetylcholine at 4; wash at 5. (*b*) Effect of arecoline (moderate concentration): 10^{-5} at 1; 10^{-4} at 2; wash at 3. (*c*) Effect of 2,268 F: 10^{-4} at signal. (*d*) Effect of 2,268 F + atropine: 10^{-4} atropine at start; 10^{-4} atropine + 10^{-5} 2,268 F at 2; wash at 3.

Since the effect of 2,268 F is not antagonized by atropine it cannot be regarded as typically muscarine-like (cf. Dale, 1914) any more than that of acetylcholine. The response to arecoline at 10^{-4} appears to contradict this conclusion, but it should be remembered that the pharmacology of arecoline itself is not very clearly understood and that its paralysant action in this particular tissue may perhaps be only a specific narcotic effect.

Other inactive compounds included caffeine, histamine, morphine, quinine, and tyramine, all at 10^{-4}—10^{-3}.

DISCUSSION

The use of annelid muscle as a means of testing anthelminthic potency was originally adopted by Trendelenberg (1916) for two reasons; first, the technical difficulty of exposing *Ascaris* muscle free from cuticle, and, secondly, because he believed that earthworms probably respond to santonin, with which he was primarily concerned, in much the same manner as *Ascaris* itself does. In our experiments, however, we find that santonin, which violently stimulates the denervated body-wall muscle of the earthworm, actually paralyses *Ascaris* muscle, usually after transient preliminary stimulation. This apart, the substitution of annelid for nematode material could hardly be justified in the absence of positive proof that members of the two phyla were similarly affected by a wide range of diverse chemical substances. No such evidence has been forthcoming, and, indeed, the extensive experiments of Lamson and Ward (1936) and the present work point to a wholly contrary conclusion. It is, of course, true that a number of phenolic anthelminthics of high reputation act similarly upon earthworms and roundworms, but these belong to the group of "general protoplasmic poisons" and have toxic actions upon most, if not all, kinds of animal tissues.

Even among parasitic helminths as a whole, differences between species in respect of response to different drugs are well known. It is therefore not surprising that such differences should also exist between these parasites and free-living Annelida. In point of fact, the responses of exposed *Ascaris* muscle differ in many ways from those observed in studies of annelid materials—

for example, earthworm body-wall (Wu, 1939; and our own observations mentioned below), earthworm gut (Ambache, Dixon, and Wright, 1945; Millott, 1943; Wu, 1939), lugworm body-wall (Wu, 1939), lugworm gut (Wells, 1937), and leech body-wall (Führer, 1918; Minz, 1932).

In the absence of eserine, we found that both *Ascaris* and earthworm muscle responded by mild stimulation to acetylcholine at *ca* 10^{-6}, but whereas eserine was without influence on *Ascaris* muscle it showed a weak acetylcholine-like action upon earthworm muscle at 10^{-6} and also sensitized it to the action of acetylcholine itself. Moreover, the effects of acetylcholine upon earthworm muscle diminished with time, a phenomenon not observable with *Ascaris*. These results provide suggestive evidence for the presence of cholinesterases in the earthworm and their absence from *Ascaris*. Furthermore, although small concentrations of nicotine produce acetylcholine-like effects in both tissues, higher concentrations (10^{-4}; Wu, 1939) antagonize the effect of acetylcholine in the earthworm but not in *Ascaris* (even at 10^{-3}). Pilocarpine, while provoking no response in *Ascaris* muscle, produces a slight stimulation in earthworm muscle at 10^{-6}—10^{-5}, changing to a depressant effect at 10^{-3}, which suggests that this tissue, again unlike that of *Ascaris*, is susceptible to the action of muscarine-like drugs. Further, although atropine (10^{-5}) has very little action upon earthworm muscle by itself, it does in part abolish the action of acetylcholine, which again indicates that the action of the latter on earthworm muscle, unlike that on *Ascaris* muscle, includes muscarine-like as well as nicotine-like components. Adrenaline at 10^{-5} had a weak and transient stimulant effect upon our earthworm preparations but was inert at 10^{-6}. Ephedrine (10^{-4}) produced an effect closely resembling that of adrenaline, but neither drug showed activity towards *Ascaris* muscle. These differences, added to numerous differences previously noted (Baldwin, 1943, 1948) between "sausage" preparations of *Ascaris* and earthworm muscle, and between intact specimens of the two species (Lamson and Ward, 1936), establish real and far-reaching differences between the two organisms.

Quite apart from the physiological and pharmacological differences noted here, the properties of the nematode cuticle have to be reckoned with. As our preliminary experiments showed, acetylcholine, arecoline, choline, cocaine, nicotine, pelletierine, and santonin pass slowly or not at all through the cuticle of *Ascaris*. Among these only arecoline, nicotine, and santonin had any action upon our original "sausages," and it is worthy of note that their action was far more rapid and powerful upon "anterior sausages" than upon those from the region immediately in front of the genital pore. This, no doubt, is because the cuticle is much thinner in the anterior region. But acetylcholine, choline, cocaine, and pelletierine have no action at all upon "sausages" even when applied at high concentrations to the anterior region, despite the thinness of the cuticle, and it is only when the muscle is directly exposed to the action of the drugs in question that any response can be observed. It may be presumed, therefore, that these compounds fail entirely to penetrate the cuticle.

Every one of these substances evokes more or less profound responses in earthworm muscle, and there can be no doubt that the cuticular layer of the nematodes constitutes a real and formidable barrier to the penetration of many substances, among them a number of compounds of established anthelminthic efficacy. Since there is nothing in the ordinary preparations of earthworm body-wall to offer a similar barrier to penetration, the use of earthworm body-wall can yield no information about the ability of a compound to penetrate the nematode cuticle; and such ability is necessary for anthelminthic activity.

The use of earthworm muscle (and probably other annelid material as well) in anthelminthic studies is therefore to be condemned on two main grounds: these are, first, that important physiological and pharmacological differences exist between annelid and nematode muscles, and, secondly, that earthworm preparations fail to reproduce any of the important effects attributable to the nematode cuticle, which plays an important part in determining the resistance or the susceptibility of parasitic nematodes to drugs of physiological or pharmacological interest.

Summary

1. The reactions of exposed *Ascaris* muscle to a number of compounds of physiological and pharmacological interest have been studied.

2. The muscle is stimulated by acetylcholine but appears to contain no cholinesterases.

3. The effects of acetylcholine are imitated by choline, nicotine, and barium ions, and are antagonized by tubocurarine, strychnine, and cocaine, but not by excess of nicotine.

4. The effects of acetylcholine are also imitated by 2,268 F, but atropine does not antagonize the effects of either.

5. Adrenaline, atropine, caffeine, ephedrine, ergotoxin, eserine, histamine, morphine, pilocar-

pine, quinine, and strychnine have no direct action on *Ascaris* muscle.

6. The cuticle acts as a barrier to the penetration of many compounds of physiological and pharmacological interest, including some substances possessing anthelminthic activity.

7. There are thus many important differences between *Ascaris* muscle and the muscles of annelids, and the use of the latter as test materials for the detection of anthelminthic potency is to be condemned.

One of us (V. M.) is indebted to the Agricultural Research Council for a grant, during the tenure of which the greater part of this work was carried out. We are also indebted to Dr. W. Feldberg for gifts of tubocurarine chloride and 2,268 F as well as for much valuable discussion. Our thanks are also due to Sir Henry Dale for a gift of 2,268 F, and to Professor E. Verney and Dr. C. F. A. Pantin, who read and criticized the manuscript. We also wish to thank the manager of the St. Edmundsbury Co-operative Bacon Factory, who kindly made the arrangements for our regular supplies of freshly collected *Ascaris*.

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THE ACTION OF HISTAMINE ON HYDROCHLORIC ACID AND PEPSIN SECRETION IN MAN

BY

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(Received January 6, 1949)

Since the demonstration by Popielski (1920) it has been widely recognized that injection of histamine into man and animals causes a copious flow of gastric juice of high acidity. There is, however, considerable difference of opinion as to whether the liberation of pepsin into the stomach is simultaneously augmented. Gaddum (1948), for instance, in a review says that the juice is "poor in enzymes," and Kahlson (1948) makes a similar statement. This view results largely from experiments on dogs and cats in which a variety of gastric pouches and fistulae were used (see, e.g., Vineberg and Babkin, 1931; Bowie and Vineberg, 1935). Alley (1935) has even postulated an inhibitory effect of histamine on pepsin secretion. More recently, however, Bucher, Ivy, and Gray (1941) have claimed that pepsin is increased in the stomach of the dog after histamine injection; this work has been strongly criticized by Babkin (1944). In man, a number of clinical studies (e.g., Polland, 1932) suggested a stimulation of pepsin output after histamine, and Toby (1937a) found that the peptic content of human gastric juice was as high as that produced by stimulation of the vagus in the dog. Nevertheless, Toby (1937b) was unwilling to accept the idea that histamine stimulates pepsin secretion in the human stomach, and took the view that the increased pepsin found represents a washing out of pepsin as a result of the increased flow of fluid.

In this paper we report the result of a series of experiments on human subjects.

METHODS

The subjects, with the exception of two patients with gastric ulcer, were healthy medical students, male and female, aged 20–25 years. All the experiments were performed after a 12-hour fast. After a Ryle tube had been passed, continuous aspiration of the stomach contents was carried out, the receiver being changed every fifteen minutes. To reduce dilution and contamination, saliva was expectorated as completely as possible. The receiving flasks were surrounded with ice so that the samples were cooled as they were collected; after measurement and the recording of the presence of bile and excess mucus, the fluids were filtered through pleated paper and kept in the refrigerator before and after filtration and between estimations. Few samples were markedly stained with bile pigment and these were rejected.

Free and total acid were estimated by Töpfer's method (dimethyl yellow and phenolphthalein) and pepsin by digestion of 2 mg. edestin dissolved in 2 ml. of *N*/30 HCl (*p*H 1.6). Since the series of twofold dilutions employed by Fuld (see Cole, 1933) were found to cover an unsuitable range of peptic activities the quantities of gastric juice taken were modified as shown in Table I. The tubes containing the mixtures of gastric juice and edestin were placed in a water-bath kept at 16° C., and after 30 min. 1 ml. of saturated sodium chloride solution was added and the contents of the tubes thoroughly mixed; the last tube to show complete digestion (i.e., the absence of a cloud of unchanged edestin) was noted and the activity in pepsin units (the number of mg. of edestin digested in 30 min. at 16° C. by 1 ml. of juice) recorded. It will be

TABLE I

DILUTIONS OF GASTRIC JUICE USED IN PEPSIN ESTIMATIONS: 2 ML. 0.1 PER CENT EDESTIN IN *N*/30 HCl WERE ADDED TO EACH TUBE

Tube number	ml. gastric juice diluted 1/10 with *N*/30 HCl	ml. *N*/30 HCl	Pepsin units per ml.	Dilution of original gastric juice
1	1.00	0.00	20	1 in 30
2	0.50	0.50	40	1 in 60
3	0.33	0.67	60	1 in 91
4	0.25	0.75	80	1 in 120
5	0.20	0.80	100	1 in 150
6	0.16	0.84	125	1 in 187
7	0.13	0.87	154	1 in 230
8	0.10	0.90	200	1 in 300
9	0.05	0.95	400	1 in 600

noted that, except in tube 1, the dilution attained was greater than 50; according to Bucher, Grossman, and Ivy (1945) this is necessary in order to overcome the effect of inhibitors present in the juice. It seems worthy of mention that this dilution would not be attained in experiments such as those of Toby (1936, 1937) in which Mett's method was used. It is doubtful whether full peptic activity was measured under these conditions (see Le Veen, 1946).

At least two collections were made over quarter-hour periods before injection of 1.5 mg. histamine acid phosphate (=0.54 mg. histamine) subcutaneously, the same dose being used in all experiments. In a number of experiments 1 ml. of normal saline (0.9 g. NaCl/100 ml. H_2O) was injected, and in two experiments histamine was administered after a prolonged period of aspiration of the stomach contents (Table II).

Results

The findings in fourteen experiments are shown in Fig. 1. Since no difference between the response to histamine of the ulcer patients and of the normal subjects was observed, all results have been grouped

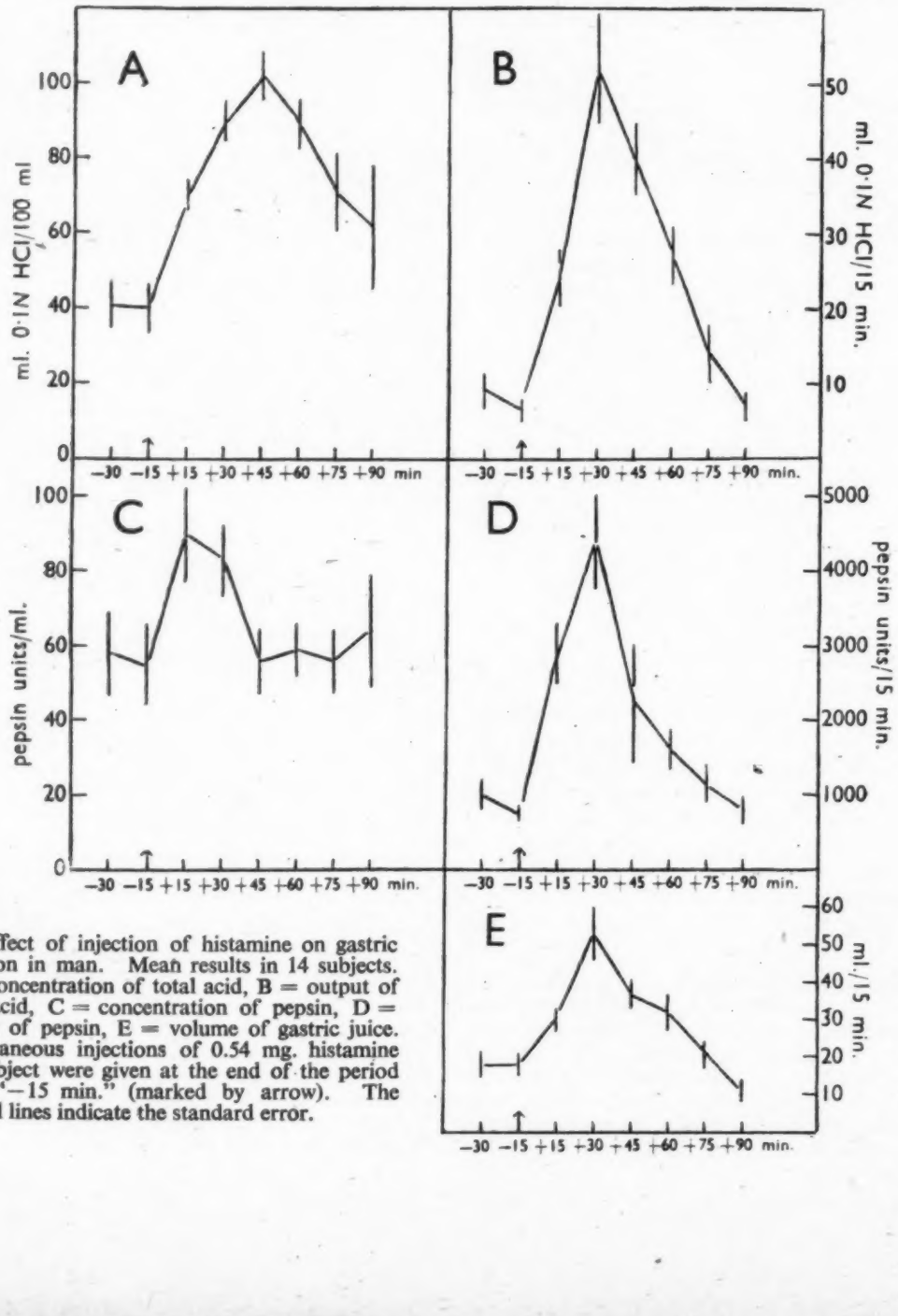

FIG. 1.—Effect of injection of histamine on gastric secretion in man. Mean results in 14 subjects. A = concentration of total acid, B = output of total acid, C = concentration of pepsin, D = output of pepsin, E = volume of gastric juice. Subcutaneous injections of 0.54 mg. histamine per subject were given at the end of the period called "−15 min." (marked by arrow). The vertical lines indicate the standard error.

together. It will be seen that in the first and second quarter-hour periods following the histamine injections, a moderate rise in pepsin concentration occurred concurrent with the rise in acid concentration. The rise in pepsin *output*, however, was more striking, reaching nearly four times the resting level in half an hour, and then declining to the resting level in the next 1¼ hours.

The "fasting contents" (=the gastric juice removed from the stomach immediately after passing the tube) were usually of considerable volume and their pepsin contents were high (Table II). It was

TABLE II

EFFECT OF INJECTION OF NORMAL SALINE ALONE, AND OF HISTAMINE INJECTION AFTER PROLONGED ASPIRATION, ON PEPSIN SECRETION IN HEALTHY SUBJECTS

No. of period (15 min.)	Subject 1 Pepsin units		Subject 2 Pepsin units		Subject 3 Pepsin units	
	per ml.	per 15 min.	per ml.	per 15 min.	per ml.	per 15 min.
"Fasting" juice	60	(6,120)	40	(3,000)	—	—
1	60	650	40	400	20	500
2	100	1,500	40	1,320	20	560
3	100	1,050	40	1,080	20	360
1 ml. normal saline injected						
4	100	1,600	20	600	40	960
5	100	1,050	20	600	20	440
6	100	1,300	20	400	20	400
7	100	1,050	20	600	40*	1,400*
8	—	—	80*	2,400*	60	6,300
9	—	—	80	5,600	—	—

*Injection of 0.54 mg. histamine per subject at beginning of these periods.

therefore thought undesirable to make comparison between the composition of the "fasting contents" and that of the juice after histamine injection, as Toby (1937b) seems to have done. In our experiment, at least two 15-minute samples were taken before injection of histamine. Table II shows that pepsin concentration and pepsin output remained reasonably steady under basal conditions. Pepsin output in some of the subjects was high. Table II shows also that an injection of normal saline had no or very little effect.

In Table III, the mean concentrations of pepsin and the outputs in the half-hour periods before and after injection of histamine are summarized. They show a rise in pepsin concentration which is significant ($t = 2.455$, $P<0.02$) and a rise in output which is highly significant ($t = 6.344$, $P<0.001$). It may be noted that the rises shown in subjects 2 and 3 (Table II) after prolonged aspiration were greater than those shown in Fig. 1; it is doubtful, however, whether any significance should be attached to these observations which were only two in number.

TABLE III

COMPARISON BETWEEN PEPSIN CONCENTRATION AND OUTPUT DURING THE HALF-HOUR PERIOD BEFORE AND THE HALF-HOUR PERIOD AFTER THE INJECTION OF 0.54 MG. HISTAMINE

	Pepsin units ± S.E. of mean of 14 observations	
	Mean concentration (units/ml.)	Mean output (units/30 min.)
Before histamine	55 ± 10	1894 ± 333
After histamine	90 ± 10	7381 ± 802

Discussion

Notwithstanding a large volume of published work and Babkin's (1944) detailed discussion of the subject, there is no general agreement as to whether histamine stimulates the secretion of pepsin into the stomach. In the dog, surgically prepared in various ways for the collection of gastric juice, there seems to be little doubt that sham-feeding and pilocarpine (Toby, 1936) are more potent stimuli than histamine, while Goodman (1938) showed that choline esters and pilocarpine were more efficacious than histamine, which nevertheless caused an increase in peptic output. Babkin (1944) and others have argued that since the histamine-stimulated secretion of pepsin may be low and since repeated injections may cause a further fall in concentration, the histamine secretion represents only a washing out of pepsin from the gastric pits. Goodman (1938), however, found a rise in pepsin on repeated injection, while Bucher and Ivy (1941) have criticized the deductions made from the "double histamine" method (see, e.g., Gilman and Cowgill, 1931), and Bucher, Ivy, and Gray (1941) suggest that histamine, like such substances as pilocarpine, should be regarded as a stimulant for pepsin. More recently, Björkman, Norden, and Uvnäs (1943), using anaesthetized cats with the pylorus ligated, failed to find a rise in pepsin activity and indeed found a decline after histamine injection, whereas vagal stimulation caused a marked rise. They considered pepsin concentration only and not the total output. It would thus appear that in animals the gastric secretion produced by histamine has a low concentration of pepsin and that, with the greatly increased flow of fluid produced by histamine, the concentration may fall although the total output may be greater than the "resting" output.

It may well be that a species difference exists between the action of histamine on the human stomach and that of the cat and the dog. This difference may be connected with the finding (Bloomfield and Polland, 1933) that a steady flow of gastric juice of fairly high peptic activity appears to occur in the fasting state in man. We also have observed in all our experiments comparatively high peptic activity in "resting" samples, and it is clear that this fact tends to mask the response to histamine if concentration and not total output is considered. It is therefore even less easy to accept Babkin's "washing out" hypothesis in man, since a fall in pepsin concentration would be expected to result from the greatly increased flow of juice which follows all injections of histamine. Toby (1937b), using a high dose of histamine (1 mg. per subject), did find such a fall, but failed to do so at a lower dosage (0.5 mg.) ; with both doses, however, pepsin *output* was increased. In none of our experiments, in which 0.54 mg. of histamine was given, was a fall of pepsin concentration observed. A probable explanation of Toby's results is as follows : Wang (1936) obtained a maximum secretion of acid (139 m. equiv./l., equivalent to an acidity of 139 ml. 0.1*N* hydrochloric acid/100 ml.) and fluid by the injection of 1 mg. of histamine into human subjects. Our figures for acid secretion with a lower dose, like those of Toby with the lower dosage, are less than this. If at the higher dosage the rates of fluid and acid formation increased more than the rate of pepsin secretion, an apparent dilution would be the result. This assumption receives further support from the findings of Blakeley and Wilkinson (1933), who at a lower dosage still (0.25 mg. per subject) found an increase of pepsin output *and* concentration which was equal to or often greater than that produced by 6 mg. pilocarpine.

It seems clear that pepsin secretion is more easily provoked in man than in animals. Nevertheless great weight has been attached to the results of those animal experiments which indicated a low peptic secretion and less attention paid to the very extensive series of experiments carried out on human subjects by, e.g., Osterberg, Vanzart, Alvarez, and Rivers (1936), who concluded that human peptic glands are stimulated by histamine. Our results are in agreement with their findings. It may be mentioned that many authors (e.g., Ihre, 1939) have only considered pepsin concentration and not output. It is probable indeed that too great consideration of pepsin concentration, which may not be markedly increased, is the root cause of the differences in the interpretation of results by various authors.

Summary

In a series of fourteen experiments on twelve normal subjects and two with gastric ulcers, it was found that injections of 0.54 mg. histamine caused a moderate rise in mean pepsin concentration concurrent with the rise in acid concentration and fluid volume ; there was, however, a marked rise in pepsin output as compared with that in the previous resting period.

We are indebted to those medical students of the University of Bristol who so kindly submitted themselves for experimentation.

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THE EFFECT OF ANTIHISTAMINE SUBSTANCES ON GASTRIC SECRETION IN MAN

BY

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(Received January 6, 1949)

At the time when the experiments to be described here were started, there were few reports regarding the action of antihistamine substances on gastric secretion, and these were contradictory. Thus Loew, MacMillan, and Kaiser (1946) found the response to histamine in three out of four dogs reduced by benadryl, and McGavack, Elias, and Boyd (1946) reported a depressant effect of benadryl on the gastric acidity following alcohol administration to normal human beings. On the other hand, Sangster, Crossman, and Ivy (1946) found no effect of benadryl on histamine-stimulated secretion in dogs, while McElin and Horton (1946) obtained variable results in patients with multiple sclerosis, and Moersch, Rivers, and Morlock (1946) adduced evidence that gastric acidity usually *increased* after the use of benadryl. It seemed therefore of interest to investigate further the action on gastric secretion in human subjects of the three antihistamine substances in common clinical use. Since previous results (Ashford, Heller, and Smart, 1949) indicated that pepsin secretion is also stimulated by histamine, the effects of these substances on both acid and pepsin output were examined.

Methods

These have been described in a previous paper (Ashford, Heller, and Smart, 1949). The subjects, with two exceptions, were healthy medical students of both sexes, and the same dose of histamine acid phosphate (1.5 mg. = 0.54 mg. base) was given by subcutaneous injection throughout. Each subject was used twice with an interval of at least one week between the experiments: on one occasion histamine was given followed or preceded by a control injection of normal saline (0.9 g. NaCl/100 ml. H_2O); on the other the antihistamine substance was given by subcutaneous injection either before or after the injection of histamine. Controls were performed also, in which either the saline solution alone or a solution of an antihistamine substance alone was injected.

The antihistamine substances studied and the doses used were:

1. β-dimethylaminoethyl benzhydryl ether hydrochloride ("Benadryl"). 50 mg. (= 44 mg. base) in 1 ml. normal saline per subject.

2. N-phenyl-N-benzylaminomethyl imidazoline sulphate ("Antistin"). 100 mg. (= 85 mg. base) in 2 ml. normal saline per subject.

3. N-*p*-methoxybenzyl-N-dimethylaminoethyl-2-aminopyridine maleate ("Neoantergan" = "Anthisan"). 100 mg. (= 72 mg. base) in 1 ml. normal saline per subject.

Results

Figs. 1, 2, and 3 compare the secretion of free hydrochloric acid, total hydrochloric acid, and pepsin respectively in the stomachs of healthy subjects who received a subcutaneous injection of histamine and of an antihistamine substance in one experiment and a subcutaneous injection of histamine only in another. Concentrations of free acid, total acid, and pepsin are shown in Figs. 1A, 2A, and 3A respectively. Output of free acid, total acid, and pepsin per 15 min. are shown in Figs. 1B, 2B, and 3B respectively.

Ten experiments with antihistamine substances were performed on five groups of two subjects. Group I consisted of two subjects to whom 50 mg. of benadryl per person had been administered 15 min. before the standard dose of histamine. In Group II 50 mg. benadryl was injected 30 min. after histamine. The subjects in Group III received 100 mg. antistin 15 min. before histamine. Group IV and V each comprised two subjects who received 100 mg. anthisan (=neoantergan) 15 min. after histamine. The points in Figs. 1, 2, and 3 marked I, II, III, IV, and V represent mean values for the gastric secretion in the respective groups during the period of 15 min. before and five periods of 15 min. after the injection of histamine. It will be seen that

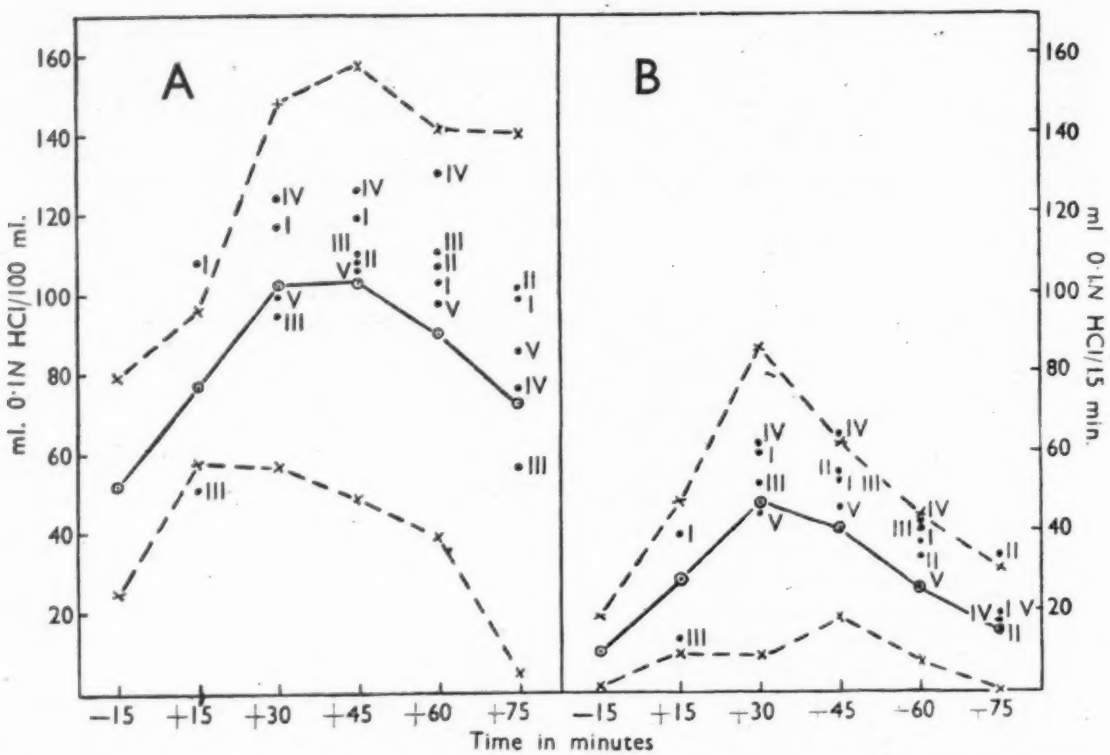

FIG. 1.—Effect of injections of antihistamine substances on histamine-provoked *free* hydrochloric acid secretion. A = ml. 0.1 *N* HCl/100 ml. B = ml. 0.1 *N* HCl/15 min. Each of the points accompanied by a Roman numeral represents the mean response to histamine plus antihistamine substance in each group of two subjects (for definition of groups see text, p. 157). ⊙——⊙ = mean line which represents the average response to histamine *alone* in the 10 subjects investigated. ×– – –× = the 1/40 Fiducial Limit for the average response of the subjects, calculated by multiplying the average range in the response in each group by a factor which transforms the average range into 1.69 times the standard deviation (see Herdan, 1948). 0.54 mg. histamine per subject was injected at the end of the period marked as "−15 min."

the points in Figs. 1 and 2 with few exceptions lie above the line indicating the mean response to histamine alone (which was calculated by pooling the results of the 10 control experiments on the same subjects). There was therefore no evidence that the concentration or output of hydrochloric acid decreased after the injection of the antihistamine substances. The distribution of the points in Fig. 3 is somewhat different in that a number of pepsin values are lower than the mean figures for the control experiments, though they are not low enough to reach the broken line which represents the Fiducial Limit of 1/40 for average results of two subjects. These decreases cannot therefore be regarded as significant. It will be noted, however, particularly in Fig. 3A, that quite a number of pepsin values are significantly *higher* than the mean level in the control experiments, suggesting that in some instances histamine plus antihistamine produced more pepsin than histamine alone. Figs. 1 and 2 indicate that the same applies to hydrochloric acid secretion. Thus in our series of experiments antihistamine substances not only failed to decrease histamine-provoked gastric secretion but, on the contrary, occasionally increased it.

The Table shows the results of experiments on normal subjects who received subcutaneous injections of the standard doses of antihistamine substances (100 mg. anthisan or 100 mg. antistin or 50 mg. benadryl per subject) alone and also the results of control experiments (subcutaneous injection of normal saline). It shows, first, that acid and pepsin secretions *before* the injection were much the same in the two series, and, secondly, that the doses of antihistamines used had no pronounced effect on "resting" gastric secretion—i.e., the secretion of hydrochloric acid and pepsin was neither considerably depressed nor considerably raised.

TABLE

COMPARISON BETWEEN THE EFFECT OF INJECTION OF ANTIHISTAMINE SUBSTANCES AND OF 0.9 PER CENT NaCl SOLUTION ON THE GASTRIC SECRETION OF NORMAL FASTING SUBJECTS. THE FIGURES SHOWN AS "BEFORE INJECTION" ARE MEAN VALUES FOR THREE 15-MIN. PERIODS, THOSE AS "AFTER INJECTION" ARE MEAN VALUES FOR FOUR 15-MIN. PERIODS. THE FIGURES IN BRACKETS ARE THOSE OBTAINED IN THE CONTROLS—I.E., AFTER THE INJECTION OF 0.9 PER CENT NaCl SOLUTION

Subject	Antihistamine injected s.c. per subject	Secretion collected before or after injection	ml. 0.1 *N* HCl/100 ml.		ml. 0.1 *N* HCl/15 min.		Pepsin units	
			free	total	free	total	per ml.	per 15 min.
To.	100 mg. anthisan	Before	30 (18)	53 (54)	3.6 (2.3)	6.3 (7.0)	100 (100)	1200 (1275)
		After	55 (29)	72 (53)	12.0 (3.5)	15.5 (6.6)	110 (100)	2150 (1220)
		Difference	+25 (+11)	+19 (−1)	+8.4 (+1.2)	+9.2 (−0.4)	+10 (0)	+950 (−55)
Sp.	100 mg. anthisan	Before	20 (16)	30 (30)	5.4 (4.9)	8.0 (9.1)	60 (40)	1620 (1200)
		After	27 (9)	38 (20)	6.5 (2.5)	9.3 (2.5)	70 (20)	1740 (550)
		Difference	+7 (−7)	+8 (−10)	+1.1 (−2.4)	+1.3 (−6.6)	+10 (−20)	+120 (−650)
Du.	100 mg. anthisan	Before	19 (24)	39 (37)	2.3 (1.2)	4.6 (1.9)	20 (40)	235 (200)
		After	6 (10)	24 (28)	0.3 (1.0)	1.5 (2.7)	60 (40)	270 (255)
		Difference	−13 (−14)	−15 (−9)	−2.0 (−0.2)	−3.1 (+0.8)	+40 (0)	+35 (+55)
El.	50 mg. benadryl	Before	55 (58)	67 (75)	14.0 (3.5)	17.0 (4.5)	150 (125)	3850 (750)
		After	55 (83)	70 (101)	6.8 (9.6)	8.5 (11.3)	130 (125)	1590 (1540)
		Difference	0 (+25)	+3 (+26)	−7.2 (+6.1)	−8.5 (+6.8)	−20 (0)	−2260 (+790)
Ew.	100 mg. antistin	Before	18 (12)	29 (15)	6.0 (2.7)	9.7 (4.7)	10 (20)	355 (430)
		After	14 (20)	25 (28)	3.2 (4.4)	6.2 (6.2)	20 (30)	510 (600)
		Difference	−4 (+8)	−4 (+13)	−2.8 (+1.7)	−3.5 (+1.5)	+10 (+10)	+155 (+170)

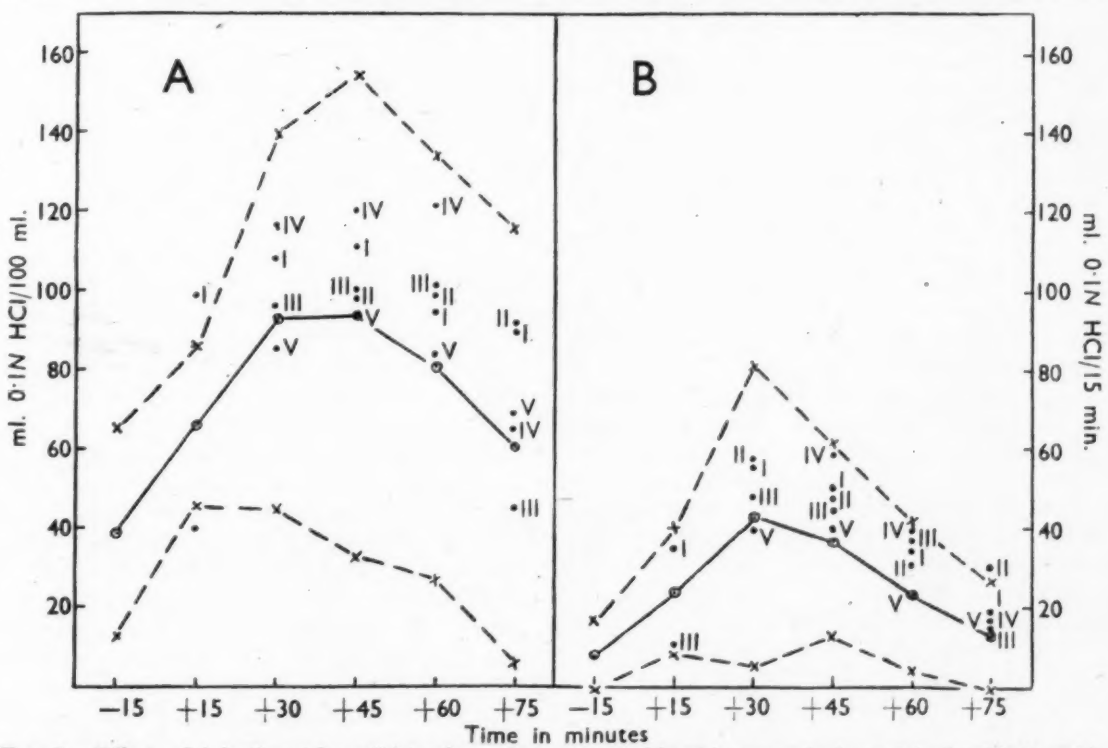

FIG. 2.—Effect of injection of antihistamine substances on histamine-provoked *total* hydrochloric acid secretion. A = ml. 0.1 *N* HCl/100 ml. B = ml. 0.1 *N* HCl/15 min. For further description see Fig. 1.

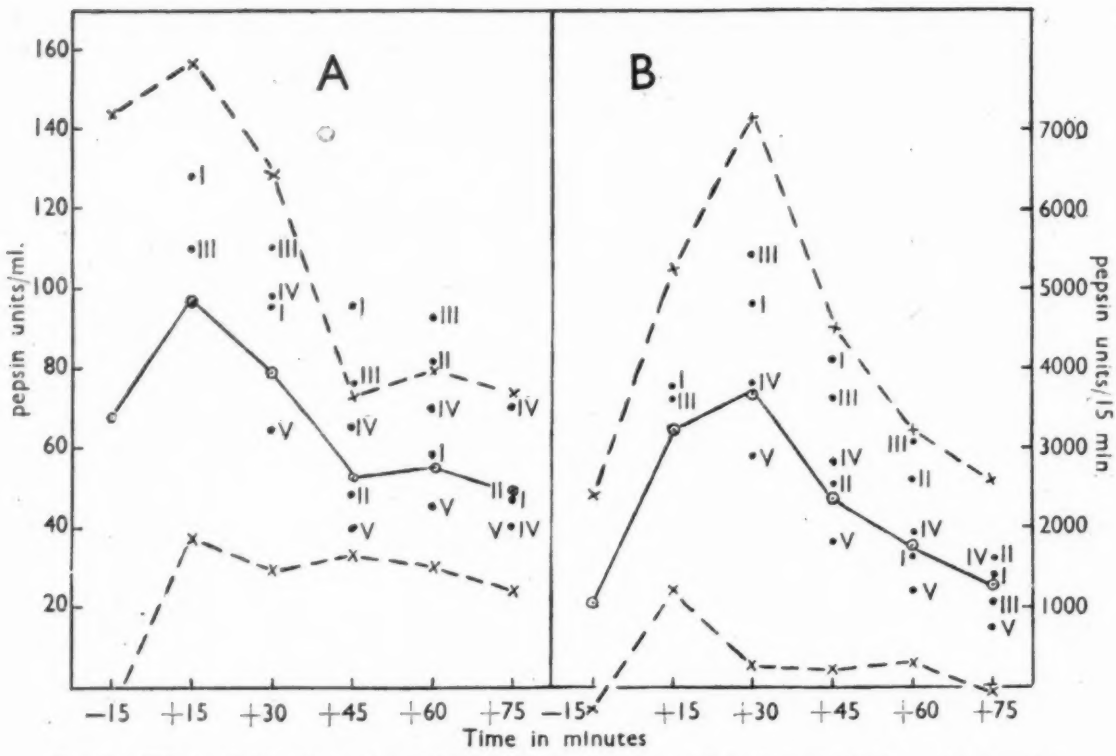

FIG. 3.—Effect of injection of antihistamine substances on histamine-provoked pepsin secretion. A = pepsin units/ml. B = pepsin units/15 min. For further description see Fig. 1.

Two experiments only were performed on patients suffering from gastric ulcers. The response to 0.54 mg. histamine injected subcutaneously was first investigated. Anthisan in doses of 100 mg. three times daily was subsequently given by mouth for 17 days to patient Hi. and for 20 days to patient Da. and the histamine test then repeated. The response of patient Hi. was found to be almost identical with that in the preliminary experiment. In the second patient (Da.) the acid concentration did not reach higher values than on the first occasion but remained at the maximum level for a longer period of time (60 as compared with 15 min.). Clinically patient Hi. showed no improvement during or immediately after the "treatment" with anthisan. Patient Da. was definitely worse, a finding which was borne out by radiological re-examination after the administration of anthisan had been terminated. It was therefore thought unjustifiable to prolong the experiments or to investigate the effects of antihistamine substances in other patients with gastric or duodenal ulcers.

DISCUSSION

The results presented show clearly that neither benadryl, antistin, nor anthisan in the dosages given decrease acid or pepsin secretion provoked by histamine in healthy human beings. In some of the subjects studied the output of acid and pepsin in fact rose above that resulting from the injection of histamine alone. Deutsch (1947), who administered 0.5 mg. histamine and 100 mg. "bridal" (=antergan) or 200 mg. antistin intravenously, appears to have obtained similar results, as also did Moersch and his co-workers (1946) in patients suffering from duodenal ulcer. Gordonoff (1948), in a small series of experiments on healthy subjects injected with 50 mg. neoantergan and 0.5 mg. histamine, failed to observe an increase of hydrochloric acid secretion.

Administration to healthy subjects of 50 mg. benadryl, 100 mg. antistin, or 100 mg. anthisan alone had no pronounced effect in our series of experiments. Doran (1947) and Deutsch (1947), however, using higher doses of antihistamine substances reported increases in hydrochloric acid concentration.

The results of these "acute" experiments are in apparent contrast with those of McGavack *et al.* (1946), who administered 200–400 mg. benadryl by mouth daily to healthy subjects for periods of two to six weeks and observed a marked depression of acid secretion. Their results are not in accord with

our findings in two ulcer patients who had received 300 mg. anthisan by mouth daily for periods of 17 and 20 days : neither a reduction of the high acid secretion nor any amelioration of clinical symptoms was obtained.

It would thus appear from our results that beneficial effects of antihistamine substances on patients suffering from gastric or duodenal ulcers may not be expected. On the contrary, the administration of these substances would seem to be contraindicated in these and similar conditions.

It is perhaps surprising that the antihistamines which antagonize all other actions of histamine whether stimulant or depressant (Dews and Graham, 1947 ; Gaddum, 1948) have no effects on the action of histamine on glandular tissues. However, since the mechanism of the action of histamine on the gastric glands is as yet imperfectly understood, it would seem unprofitable at present to speculate on the reasons for the failure to influence this particular action.

Summary

1. Subcutaneous injection of 50 mg. benadryl, 100 mg. antistin, or 100 mg. anthisan failed to lower the concentration and output of hydrochloric acid and pepsin in 10 healthy subjects to whom 0.54 mg. histamine had been administered ; on the contrary, an increase of gastric secretion was noted in some subjects.

2. Injection of the same doses of the antihistamine substances alone produced no marked alteration of acid or pepsin secretion.

3. Two patients with gastric ulcers were given 300 mg. anthisan by mouth daily for 17 and 20 days. Gastric secretion of acid was not reduced after these periods and the clinical state of the patients did not improve.

4. It is suggested that the use of antihistamine substances is contraindicated in patients suffering from gastric ulcer and similar conditions.

We are indebted to Dr. R. Wien of May and Baker, Ltd., for a supply of "Anthisan," to Dr. J. Stanley White, of Parke, Davis and Co. for a supply of "Benadryl," and to Messrs. Ciba Limited for a supply of "Antistin." We are grateful to those medical students of Bristol University who so kindly submitted themselves for experimentation.

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Brit. J. Pharmacol. (1949), **4**, 162.

THE ACTION OF SODIUM THIOCYANATE ON CARDIAC OUTPUT

BY

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(Received February 2, 1949)

Sodium thiocyanate has been introduced with variable success into the treatment of human hypertension. Its mode of action is little understood. Two series of observations indicated a possible hypothesis which might be tested experimentally: (1) the SCN anion markedly and rapidly sensitizes striped and cardiac muscle to the action of K ions (Bacq, 1942, 1947; Derouaux, 1942); (2) injection of veratrine, another substance which sensitizes mammalian tissues to K ions (Szent-Györgyi, Bacq, and Goffart, 1939; Bacq and Goffart, 1939; Bacq, 1939), induces a rapid fall of blood pressure if the vagi are active (Amann and Jarisch, 1943; Amann, Jarisch, and Richter, 1941; Jarisch and Richter, 1939). This fall of blood pressure (also called the Bezold effect) is a reflex originating in the heart itself, mainly in the left ventricle (Dawes, 1947); the afferent fibres travel in the vagi, and the fall of blood pressure is due to inhibition of the vasoconstrictor centre; the simultaneous slowing of the heart has less importance because atropine does not restore the original level of the arterial pressure (Amann *et al.*, 1941, 1943; Jarisch and Richter, 1939).

Sodium thiocyanate does not alter the blood pressure if given in reasonable amounts (Amann and Jarisch, 1943), but one might expect to observe, after injection of NaSCN, a fall in cardiac output, compensated by vasomotor action in the normal animal.

Methods

Dogs anaesthetized with chloralose were used. The blood pressure in the femoral artery was registered and the heart rate could be obtained accurately at any moment. Calculations of the cardiac output were based on the well-known Fick's principle that the cardiac output (in ml. per minute) equals the oxygen consumption (in ml. per minute) divided by the difference (in ml. per litre) between the oxygen content of arterial blood and the oxygen content of mixed venous blood. Samples of blood were collected with the usual care to avoid air contamination. Mixed venous blood was obtained directly from the right ventricle by right heart catheterization according to Cournand's technique (Cournand and Ranges, 1941), the catheter being passed along the right jugular vein. Arterial blood taken from the femoral artery was invariably found to be 95–97 per cent saturated with oxygen. Blood gas analyses were carried out in Haldane's classical apparatus. Oxygen consumption was determined by analysis of expired air collected in a Douglas bag and measured in a spirometer; the gas analysis apparatus of Haldane was used for the measurements of oxygen and carbon dioxide. Thiocyanate concentration in plasma was easily determined thanks to the colour reaction of this ion with ferric ions (Bacq and Fischer, 1947). Potassium concentration of plasma was obtained by the classical cobaltinitrite method of Leulier, Velluz, and Griffon. Sodium thiocyanate and all other substances were injected intravenously in aqueous solution, injections being made slowly (in 7 to 20 minutes).

Results

Tables I and II show that thiocyanate decreases the cardiac output down to 50 per cent if the vagi are normally active (Exps. 1, 2, 3, 11, 12). The fall of cardiac output is bigger when in the same animal the level of thiocyanate in blood is higher, but there is no strict proportionality. An effect on cardiac output is visible when thiocyanate reaches concentrations considered to be therapeutically useful and safe in human hypertensive patients (Barker *et al.*, 1941; del Solar *et al.*, 1945). The single curious exception is that of dog 8 (Table II); its cardiac output increased after injection of thiocyanate; this dog had no vagal tone since 4 mg. atropine sulphate failed to change the heart rate.

The femoral blood pressure generally remains unaltered; if it falls, it is only at the end of the experiment, when cardiac output has been considerably reduced for a long period (Exp. 1).

The oxygen consumption does not decrease and quite naturally the oxygen desaturation of the mixed venous blood increases as the cardiac output falls. Typical instances may be seen in Exps. 1 and 12.

In several animals (Nos. 2, 4, and 9) muscular tremor and spontaneous or reflex convulsions

TABLE I

Exp. number and dog's weight	Time	Experimental conditions	Oxygen consumption (ml./min.)	Oxygen arterio-venous difference in blood (ml./l.)	Cardiac output		Heart rate		Systolic output in % normal	Arterial blood pressure in mm. of Hg	SCN^- in plasma mg./100 ml.	Observations
					ml./min.	in % normal	minute rate	in % normal				
1 (13 kg.)	9.53	Normal	75	28.5	2,630	100	142	100	100	140	—	
	10.01	NaSCN, 5 mg./kg. ..										
	10.08		72	39.5	1,830	70	124	87	80	130	—	
	10.19	NaSCN, 10 mg./kg.										
	10.26		75	59	1,280	49	90	63	77	120	—	
	10.47		70	49.5	1,410	54	100	70	71	100	—	
	11.18		67	59	1,140	43	132	93	47	80	—	
2 (12.5 kg.)	9.51	Normal	74	32	2,306	100	164	100	100	145	0.6	
	9.57	NaSCN, 10 mg./kg.										
	10.01	NaSCN, 10 mg./kg.										
	10.06		71	34.5	2,070	90	164	100	90	150	12.1	
	10.33	NaSCN, 20 mg./kg.										
	10.52	NaSCN, 20 mg./kg.										
	10.59		97	57.	1,696	74	180	110	67	160	13.1	Convulsions
	11.30	NaSCN, 50 mg./kg.										
	11.38		138	106	1,305	57	160	99	58	160	20.8	
3 (19.5 kg.)	9.29	Normal	85	54.5	1,560	100	90	100	100	100	0.4	
	9.45	NaSCN, 20 mg./kg.										
	9.53		85	70	1,217	78	78	87	90	100	5.2	
	10.02	NaSCN, 40 mg./kg.										
	10.11		95	88	1,075	69	87	97	72	100	9.6	
	10.19	NaSCN, 80 mg./kg.										
	10.27		100	109	917	59	93	103	57	100	25.0	
	10.34	Both vagi cut ..										
	10.39		81	61	1,332	85	183	203	42	70	25.4	
	11.19		91	76	1,193	76	164	182	42	45	18.3	
4 (14.5 kg.)	9.15	Both vagi cut ..										
	9.45		89	30	2,980	100	168	100	100	125	0.4	
	9.54	NaSCN, 30 mg./kg.										
	10.01		81	28	2,900	97	135	80	121	135	6.7	
	10.10	NaSCN, 50 mg./kg.										
	10.18		81	28	2,900	97	128	76	128	140	13.2	
	10.28	NaSCN, 60 mg./kg.										
	10.35		81	28	2,900	97	130	77	126	140	19.8	
	11.15		90	55	1,640	55	140	83	66	120	22.3	Hyperreflectivity
5 (11.5 kg.)	9.19	Both vagi cut ..										
	9.48		102	39	2,620	100	186	100	100	155	0.4	
	9.59	NaSCN, 30 mg./kg.										
	10.06		103	31	3,330	127	186	100	127	165	8.1	
	10.17	NaSCN, 100 mg./kg.										
	10.24		104	33	3,160	120	186	100	120	180	23.1	
	11.22		110	46	2,390	91	182	98	93	200	18.3	
6 (13 kg.)	9.16	Atropine, 2 mg. ..										
	9.46		64	88	730	100	146	100	100	135	0.3	
	9.54	NaSCN, 30 mg./kg.										
	10.01		61	85	720	98	140	96	102	145	9.4	Hyperreflectivity
	10.09	NaSCN, 100 mg./kg.										
	10.16		61	79	775	106	136	93	114	155	25.5	
	11.14		60	110	550	75	130	89	84	140	23.6	
7 (17.5 kg.)	9.18	Atropine, 2 mg. ..										
	9.47		82	39	2,100	100	180	100	100	135	0.4	
	9.56	NaSCN, 30 mg./kg.										
	10.03		83	41.5	1,990	95	164	92	103	145	7.0	
	10.09	NaSCN, 50 mg./kg.										
	10.16		78	40	1,950	92	158	88	105	145	14.3	
	10.27	NaSCN, 60 mg./kg.										
	10.34		73	40	1,820	87	160	89	98	145	24.7	
	11.32		65	57	1,140	54	148	82	66	130	20.6	

TABLE II

Exp. number and dog's weight	Time	Experimental conditions	Oxygen consumption (ml./min.)	Oxygen arterio-venous difference in blood (ml./l.)	Cardiac output (ml./min.)	Cardiac output in % normal	Heart rate minute rate	Heart rate in % normal	Systolic output in % normal	Arterial blood pressure in mm. of Hg	SCN^- in plasma mg./100 ml.	Observations
8 (11.5 kg.)	9.32	Normal	51	56	920	100	96	100	100	120	0.2	
	9.38	NaSCN, 30 mg./kg.										No vagal tone
	9.45		49	41.5	1,190	129	86	90	144	135	—	
	9.50	NaSCN, 30 mg./kg.										
	9.57		56	38.5	1,450	158	87	91	174	145	13.8	
	10.04	Calcergyl,* 50 mg./kg.										
	10.15		49	34.5	1,430	156	91	95	164	135	12.4	
	10.25	Calcergyl, 50 mg./kg.										
	10.33		50	43.5	1,140	124	88	92	134	125	12.1	
	10.38	NaSCN, 80 mg./kg.										
	10.45		48	53.5	900	98	88	92	106	120	22.5	
9 (9.5 kg.)	9.37	Normal	59	39.5	1,500	100	111	100	100	150	0.3	
	9.43	NaSCN, 30 mg./kg.										
	9.50		69	49	1,400	93	103	93	100	170	9.5	Convulsions
	10.00	NaSCN, 50 mg./kg.										
	10.07		72	57	1,270	84	105	95	88	185	14.6	Convulsions
	10.13	Calcergyl, 50 mg./kg.										
	10.24		84	53.5	1,570	104	113	102	102	180	12.1	
	10.32	Calcergyl, 50 mg./kg.										
	10.40		84	53	1,585	105	106	96	110	190	13.0	
	10.52	NaSCN, 80 mg./kg.										Convulsions
	10.59		82	52	1,570	104	124	112	93	190	24.1	
10 (25 kg.)	9.30	Normal	120	57	2,110	100	125	100	100	175	0.3	
	9.39	Calcergyl, 100 mg./kg.										
	9.47		122	52.5	2,315	110	102	82	135	180	—	
	9.54	NaSCN, 30 mg./kg.										
	10.00		111	47.5	2,340	111	96	78	145	180	7	
	10.08	NaSCN, 50 mg./kg.										
	10.15		114	42	2,715	129	96	78	168	185	12.7	
	10.33	NaSCN, 80 mg./kg.										
	10.40		110	46	2,390	113	92	74	155	185	19.8	
	11.40		99	57	1,730	82	84	67	123	185	21.3	
11 (16 kg.)	9.36	Normal	85	34	2,500	100	141	100	100	130	0.3	
	9.41	NaSCN, 30 mg./kg.										
	9.48		84	39	2,160	86	107	76	114	140	6.4	
	9.54	NaSCN, 30 mg./kg.										
	10.01		90	42	2,145	86	98	70	124	140	9.7	
	10.08	NaSCN, 50 mg./kg.										
	10.15		92	47	1,955	78	101	72	109	160	16.1	
	10.21	Calcergyl, 100 mg./kg.										
	10.32		97	37	2,615	104	86	61	172	170	15.1	
12 (16.5 kg.)	9.34	Normal	87	35.5	2,465	100	188	100	100	140	0.3	14 mg. K/100 ml. serum
	9.41	NaSCN, 80 mg./kg.										
	9.48		86	48	1,785	72	128	68	106	155	12.8	Convulsions
	9.57	NaSCN, 80 mg./kg.										
	10.05		89	47	1,900	77	128	68	114	155	19.1	12.7 mg. K/100 ml. serum
	10.22	KCl, 12 mg./kg. ..										Continuous muscular
	10.56	KCl, 12 mg./kg. ..										tremor; 16.6 mg. K/
	11.03		165	70.5	2,340	95	228	121	79	145	17.7	100 ml. serum
	11.16		226	100	2,260	92	232	123	74	145	14.8	15.5 mg. K /100 ml. serum
13 (19.5 kg.)	9.28	Normal	124	23	5,380	100	176	100	100	160	0.5	11.8 mg. K/100 ml. serum
	9.36	NaSCN, 150 mg./kg.										Hyperreflectivity
	9.49		121	32.5	3,715	69	116	66	105	185	15.8	12.4 mg. K/100 ml. serum
	10.02	KCl, 5 mg./kg. ..										
	10.16		124	37	3,365	63	110	63	100	170	15.4	13.2 mg. K/100 ml. serum
	10.32	KCl, 5 mg./kg. ..										
	10.43		111	43	2,585	48	90	51	94	170	15	12.8 mg. K/100 ml. serum
	11.06		116	52	2,235	42	77	44	95	170	14.8	11.9 mg. K/100 ml. serum
	11.12	KCl, 10 mg./kg. ..										
	11.23		101	52	1,950	36	72	41	89	175	14.4	12.9 mg. K/100 ml. serum

* Calcergyl = calcium thiosulphate Labaz.

occurred, which introduced the complicating element of an increased oxygen consumption; nevertheless, the fall in cardiac output persisted; it simply failed to deepen when thiocyanate concentration increased in the blood (see beginning of Exp. 12) or was smaller than usual (beginning Exp. 9).

That the vagi (and not their afferent but their inhibitory cholinergic efferent fibres) are involved in this process is proved by the following observations: (1) if both vagi are cut in the neck before injection of thiocyanate, no significant decrease of cardiac output takes place although the concentration of thiocyanate in blood reaches high levels (Exps. 4 and 5); the final fall in Exp. 4 is of little value at the end of the experiment and was preagonic; (2) bilateral vagotomy, performed during the experiment, when the cardiac output was reduced to 59 per cent, increased this output at once, although the SCN^- concentration in blood did not decrease (Exp. 3); (3) two dogs with vagi intact but given a moderate dose (2 mg.) of atropine sulphate reacted to thiocyanate very much like the vagotomized animals (Nos. 6 and 7).*

That potassium ions are involved in the response to thiocyanate is more difficult to prove. A first attempt (Exp. 12, Table II) to increase K^+ concentration in plasma by slow intravenous injection of

* Cardiac output decreases two hours after injection of atropine because the effect of atropine wears off (Exps. 6 and 7).

TABLE III

Exp. number and dog's weight	Time	Experimental conditions	Oxygen consumption (ml./min.)	Oxygen arterio-venous difference in blood (ml./l.)	Cardiac output		Heart rate		Systolic output in % normal	Arterial blood pressure in mm. of Hg
					ml./min.	in % normal	minute rate	in % normal		
14 (19.5 kg.)	9.46	Normal	100	44	2,275	100	105	100	100	125
	10.02	Veratrine, 20 μg./kg.								
	10.04					—	—	—	—	65
	10.15	Veratrine, 20 μg./kg.								120
	10.21		98	48	2,040	90	121	115	77	95
	10.39	Veratrine, 20 μg./kg.								
	10.48		77	66.5	1,160	51	78	74	68	70
	11.36		83	54	1,540	70	108	103	66	90
15 (15 kg.)	9.36	Normal	109	38	2,860	100	88	100	100	145
	9.46	Veratrine, 26 μg./kg.								
	10.01		100	58.5	1,715	60	78	89	68	120
	10.19	Veratrine, 13 μg./kg.								145
	10.34	Veratrine, 130 μg./kg.								140
	10.46		97	85.5	1,140	40	54	61	65	70
	10.50	Atropine, 4 mg. ..								
	11.15		96	69	1,400	49	200	227	22	70
	11.21	Both vagi cut ..								
	11.39		137	60	2,280	80	236	268	29	135
16 (25.5 kg.)	9.40	Normal	108	35	3,090	100	76	100	100	150
	9.53	Veratrine, 40 μg./kg.								
	10.08		91	53.5	1,700	55	100	132	42	120
	10.24	Veratrine, 80 μg./kg.								
	10.37		99	54.5	1,810	59	87	115	51	95
	10.43	Calcergyl, 100 mg./kg.								
	10.51		97	42.5	2,280	74	178	234	31	110
17 (23.5 kg.)	9.33	Normal	162	58	2,800	100	140	100	100	150
	9.47	Veratrine, 83 μg./kg.								
	10.00		143	57.5	2,490	89	123	88	102	120
	10.17	Veratrine, 144 μg./kg.								
	10.22		113	99.5	1,130	40	96	69	59	95
	10.37		105	85.5	1,235	44	68	49	91	70
	10.42	Calcergyl, 100 mg./kg.								
	10.56		137	53.5	2,570	92	134	96	96	115
	11.04	Both vagi cut ..								
	11.15		175	56	3,120	112	196	140	80	150

KCl in a dog under the influence of SCN^- was followed by vigorous and continuous muscular twitchings which increased oxygen consumption and cardiac output. A second observation (No. 13, Table II) is at first sight more successful; after a single injection of NaSCN, which decreased cardiac output to 69 per cent of normal, several injections of KCl did not cause convulsions and brought the output progressively down to 36 per cent, although the thiocyanate concentration in the blood decreased slightly; a more careful analysis shows: (1) that the potassium level in serum was just slightly higher, (2) that the heart rate was exceptionally low, and (3) that a progressive heart failure due to SCN^- alone is a quite possible interpretation of the data.

Better evidence is given by the relief brought about by Ca ions which are known to antagonize K ions and the sensitizers to K ions (Bacq, 1939, 1947); we used supersaturated stabilized solutions of calcium thiosulphate* which are highly ionized and perfectly tolerated. A suitable dose of Ca ions injected before large amounts of thiocyanate prevents the fall of output (Exp. 10, Table II); when given after NaSCN, it restores cardiac efficiency (Exps. 9 and 11, Table II) and protects against a further administration of thiocyanate (Exp. 9, Table II); given alone, calcergyl is inactive.

In order to have the necessary data for a clear discussion, we made four observations with veratrine under similar experimental conditions (Table III). Small doses of veratrine decrease cardiac output, systolic output, and heart rate if the vagi are present. After veratrine, atropine has little effect on reduced cardiac output, although the heart rate is more than trebled in Exp. 15; calcium ions are quite effective in antagonizing veratrine action (Exps. 16 and 17), but the relief is not complete since bilateral vagotomy results in a further increase (Exp. 17).

The last important fact is a considerable increase in cardiac output following vagotomy in the dog after treatment with veratrine and atropine (Exp. 15). These observations are in close agreement with the data of Jarisch and his colleagues, showing that the efferent fibres in the vagi have, in veratrine reflex action, a much smaller importance than the afferent fibres.

Discussion

The fall of cardiac output after sodium thiocyanate does not happen in absence of the vagi and is inhibited by Ca ions just as the similar action of veratrine. Potassium ions are involved in the process as expected; but the mechanism is not yet clear. Does SCN^- increase the reaction to K ions of the sensitive elements in the ventricle? Does it act on the vagal centres? Or does it simply increase at the periphery the response of the heart to the inhibitory impulses of the efferent fibres?

The last possibility is probably correct for the following reasons: (1) atropine is nearly as effective as vagotomy in thiocyanate, but not in veratrine, poisoning; (2) thiocyanate increases remarkably the inhibitory effects of peripheral vagus nerve stimulation (Bregante, 1945, 1947; Goutier, 1948); (3) thiocyanate increases the direct effect of acetylcholine on the muscle, by a mechanism which is certainly not the inhibition of tissue cholinesterase, but most probably the potentiation of the K ions released by the action of acetylcholine (Vanremoortere, 1949); (4) thiocyanate differs from veratrine in the absence of decurarizing properties, in its lack of action on the end plate (Coppée, 1943); (5) there is no true Bezold effect with NaSCN (Amann and Jarisch, 1943); (6) our dog 8, which was insensitive to NaSCN, had practically no vagal tonus; (7) in previous experiments, two of us (Charlier and Philippot, 1947, 1948a) have observed that bilateral vagotomy in the dogs under chloralose results in irregular, generally small, changes in cardiac output. Eleven animals were used: in six, there was an increase (mean value + 10 per cent); in five, a decrease was noted (mean value −19 per cent); the highest changes recorded were +22 per cent and −27 per cent. In dogs given thiocyanate, bilateral vagotomy increases cardiac output and this increase is bigger than in normal dogs (44 per cent).

The main action of thiocyanate should be to increase the inotropic effect of the vagal tone and thus decrease the contractile power of the heart. This inotropic effect cannot exist without some chronotropic action; as a rule NaSCN decreases the dog's heart rate when the vagi are present (Exps. 1, 3, 8, 9, 11, 12, 13); Exp. 2 is an exception, and so is Exp. 4, in which no slowing of the heart should have happened in absence of the vagi. In the normal dog under chloralose, thiocyanate does not induce a fall of blood pressure. Since the cardiac output is diminished, it means that the total peripheral resistance of the blood vessels increases at the same time. A similar phenomenon—namely, decrease of cardiac output and simultaneous increase in total peripheral resistance, resulting in no change of arterial blood pressure—occurs under light anaesthesia with chloroform (Charlier and Philippot, 1948b).

The therapeutic implications of this work are serious. Thiocyanate just weakens the heart; it

* We wish to thank the "Laboratoires de recherches pharmaceutiques de l'Azote (Liége)" for a generous supply of this preparation (Calcergyl Labaz).

does not act on the kidneys or the vessels; it does not seem to change anything in the basic mechanism of hypertension; its use, which is not devoid of danger, should thus be confined to those cases where the pressure has become dangerous to the heart and is unnecessarily high for a suitable glomerular filtration; in other words, where the reaction of the organism to renal ischaemia or general sclerosis is out of proportion. The fall in cardiac output in the hypertensive patient should not be so easily compensated as in the normal dog, or the hypertrophied heart might be more sensitive. This would explain why hypotension is observed in hypertensive patients and not in normal animals.

When symptoms of thiocyanate intoxication occur, quick relief may be obtained by intravenous injection of ionized calcium salts. It is interesting to note that the human organism reacts to chronic intoxication with KSCN by liberation of Ca ions from the bones; Hinchey, Hines, and Ghormley (1947) have reported osteoporosis occurring in 2 per cent of the hypertensive patients treated with potassium thiocyanate three to six months after the start of the treatment. Cessation of treatment was always followed by relief.

One wonders if the therapeutic effects of SCN^- in hypertension might not be obtained with eserine, which increases the peripheral effects of vagal tone by a completely different mechanism.

Summary

1. NaSCN decreases the cardiac output of dogs under chloralose; this action is antagonized or prevented by bilateral vagotomy, by atropine, or by injection of Ca ions.

2. Arterial blood pressure remains normal even when the cardiac output is decreased 50 per cent by NaSCN.

3. Veratrine also decreases the cardiac output, and its action is inhibited by vagotomy and Ca ions but not by atropine.

4. Discussion shows that the action of SCN ions in normal dogs and hypertensive patients is probably due to an increase of the heart response to vagal tone; the sensitization of the heart muscle to K ions is quite sufficient to account for the action of thiocyanate.

5. The use of calcium in thiocyanate intoxication is logical and advisable.

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SOME OBSERVATIONS UPON THE PHARMACOLOGICAL ACTIVITY OF DI-*ISO*PROPYL FLUOROPHOSPHONATE

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(Received February 4, 1949)

Interest in the di-alkyl fluorophosphonates arose mainly out of the work of Adrian, Feldberg, and Kilby (1946, 1947), who showed that the most active member of the group was di-*iso*propyl fluorophosphonate, or DFP as it is usually termed. The work of others confirming and enlarging these original observations has been reviewed (Quilliam, 1947). DFP is of considerable clinical importance; for example, recent studies have shown it to be a powerful weapon for combating post-operative paralytic ileus (Quilliam and Quilliam, 1947; Grob, Lilienthal, and Harvey, 1947). The object of this paper is to record details of some of the pharmacological actions of DFP preliminarily reported by Quilliam and Strong (1947a and b, 1949).

The Isolated Rabbit Heart

Method

The hearts of rabbits were perfused with oxygenated Ringer-Locke solution by the usual Martin-Langendorff method. The duration of complete cessation of the heart beat (followed by a resumption of cardiac activity) was adopted as a means of comparing the sensitivity of preparations to acetylcholine at the various phases of each experiment. Control injections of redistilled propylene glycol, the solvent used for the DFP, were made in order to exclude sensitivity changes arising from this source. Solutions of DFP in propylene glycol, containing 10, 1.0, 0.1, or 0.01 g. per 100 ml., retained their activity for at least two months provided care was taken to keep the container firmly stoppered and to exclude entry of water to the stock solution by using carefully dried pipettes and syringes to withdraw samples of DFP solutions. For most experiments, a 1 g./100 ml. solution of DFP was used and as small volumes as possible were injected—e.g., 0.025 ml.—with a microsyringe.

Results

Action of DFP upon the heart

If more than 25 mg. of pure DFP were injected into the perfusion fluid, the heart beat ceased immediately in diastole and recovery was never seen. When 10 to 25 mg. pure DFP were administered, the substance still appeared to be very toxic and the heart was arrested at once. Occasionally, however, after an interval of five to ten minutes the heart started to beat again feebly, the amplitude being extremely small and the recovery very incomplete. Within the dosage range 2.5 to 10 mg. DFP, there was an immediate cessation of the heart beat which was usually followed by a somewhat greater degree of recovery. In the range 0.25 to 2.5 mg., DFP caused a partial and transient reduction of the amplitude of the beat from which the heart recovered fully or very nearly so in the space of two minutes (Figs. 2 and 3).

With quantities of DFP less than 0.25 mg., the transient reduction of the beat was less marked and complete recovery correspondingly accelerated (Fig. 1). Reduction of the heart rate after an injection of 0.25 mg. to 2.5 mg. DFP was conspicuous by its absence, having been seen only once in a large number of experiments, and the heart rate remained constant throughout the period during which the heart was exposed to this dosage range of DFP alone. The partial depression of the heart beat due to an injection of DFP was uninfluenced by prior treatment of the heart with sufficient atropine to render it insensitive to acetylcholine; moreover, such a heart remained unresponsive to acetylcholine after DFP, no reversal of the full effect of atropine being seen. If the heart was showing signs of returning sensitivity to acetylcholine, usually some time after treatment with atropine, then DFP tended to accelerate the return of full sensitivity to the action of acetylcholine. Control injections of 0.025 ml. of propylene glycol failed to alter the sensitivities to acetylcholine of a number of preparations.

When a simple siphon with an electronic flow recorder was used (Quilliam, 1949), no change in coronary flow was detected during any period of

action of DFP alone on the heart. In electrocardiographic studies, Knox, Quilliam, and Strong (1949) showed that the slight prolongation of the P–R interval, the transient broadening and splintering of the P or QRS complexes of small extent, and the slight changes in the amplitude of T, which occur after injections of DFP (0.25 to 0.5 mg.) into the isolated heart, could be attributed to the action of the propylene glycol used as a solvent and not to the DFP itself.

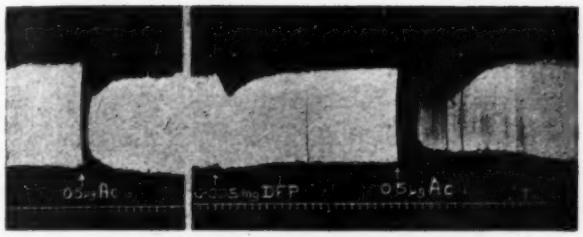

FIG. 1.—The action of 0.5 μg. of acetylcholine (Ac) upon the isolated perfused rabbit's heart before and after a very small dose of DFP (0.005 mg.) is illustrated and a marked potentiation of the effect of the second dose of acetylcholine is demonstrated. (All tracings are to be read from left to right.)

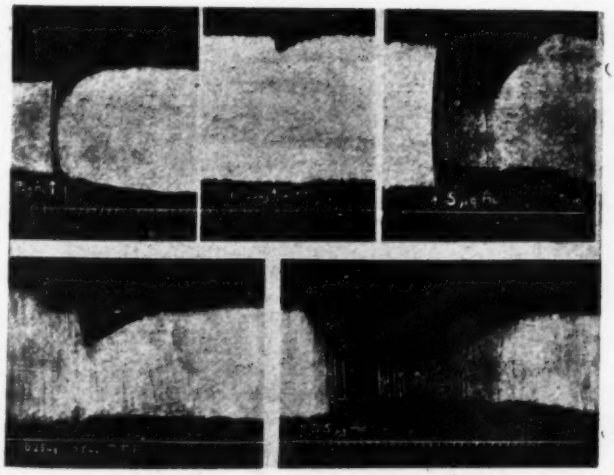

FIG. 2.—The action of 5 μg. of acetylcholine (Ac) is markedly potentiated by 0.025 mg. of DFP—i.e., partial sensitization of the heart. To sensitize the preparation fully, 0.25 mg. and then 0.5 mg. DFP were injected and afterwards the heart showed a great increase in sensitivity to acetylcholine (Ac): a dose of 0.5 μg. now gave a marked complete inhibition followed by a prolonged slowing. The sensitization was considerably greater than ten times. The slow onset of the acetylcholine effect after DFP seen in the above tracing sometimes occurred at the end of a long experiment.

FIG. 3.—In this record, an isolated rabbit heart required 50 μg. acetylcholine (Ac) to produce a marked arrest of the beat. When the transient depression caused by the injection of 0.25 mg. DFP had passed off, the effect of 0.5 μg. acetylcholine will be seen to be somewhat greater than the action of the initial 50 μg. of acetylcholine before DFP. The sensitization to acetylcholine produced in the heart by DFP is thus a little greater than one-hundredfold.

Changes in the sensitivity of the heart to acetylcholine after DFP

With small quantities—e.g., 0.005 mg.—of DFP there was a clear potentiation of the action of acetylcholine in concentrations in which it had previously had an effect (Fig. 1). With a larger dose—e.g., 0.025 mg.—of DFP the potentiation of a given dose of acetylcholine was more marked. In order to demonstrate the full sensitizing power of DFP, a still larger dose had to be injected—e.g., 0.25 mg. + 0.5 mg. DFP—after which the sensitivity of the heart was increased between ten and one-hundredfold (Fig. 2). With a dose of 0.25 mg. DFP in a fresh preparation (Fig. 3), it was often possible to obtain more than a hundredfold sensitization of the heart to acetylcholine. The action of acetylcholine after DFP was prolonged compared with its evanescent action in the normal isolated heart. The heart usually recovered completely from the partial reduction in amplitude of the beat about two minutes after an injection of 0.25 mg. DFP, and at this point the sensitization of the preparation to acetylcholine appeared to be fully developed. This sensitization was so resistant to prolonged perfusion with normal Ringer-Locke fluid that it was considered to be permanent. While there were some individual variations, maximal sensitivity was usually developed after a single injection of 0.25 mg. to 0.5 mg. DFP.

The action of DFP on the heart after eserine or prostigmine

If an isolated rabbit heart was perfused with Ringer-Locke solution containing eserine sulphate (1/100,000), the usual sensitization to acetylcholine was observed. An injection of 0.25 mg. DFP now produced but a very slight increase in the sensitivity of the preparation. When normal Ringer-Locke

fluid was restored to the heart, the removal of the eserine resulted in a marked decrease in sensitivity to acetylcholine. If the heart was then subjected to a further 0.25 mg. DFP, there was a marked increase in response to the reference dose of acetylcholine (Fig. 4).

Similar results were obtained in a fresh rabbit heart perfused with Ringer-Locke solution containing prostigmine (1/100,000); 0.25 mg. DFP now produced little change in the sensitivity of the preparation, but when normal Ringer-Locke fluid was restored the sensitivity of the heart was markedly reduced and could be enhanced subsequently by 0.5 mg. DFP.

If the sensitization produced by eserine or prostigmine was not maximal, a dose of DFP caused additional sensitization which could not be removed by perfusion with normal Ringer-Locke solution.

The action of eserine and prostigmine on the heart after DFP

If a heart was fully sensitized to acetylcholine with DFP and then perfused with eserine sulphate

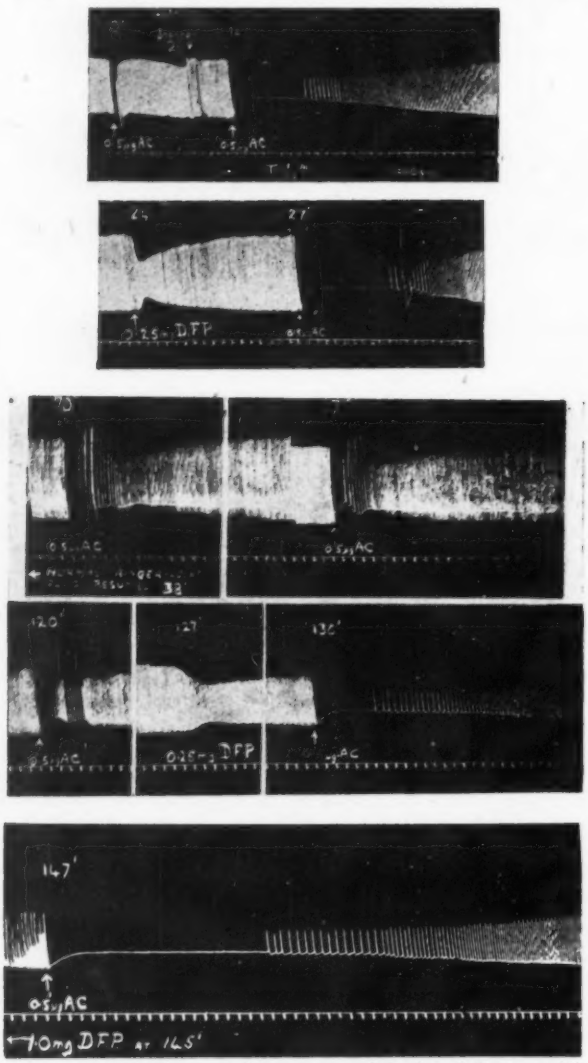

FIG. 4.—Treatment of the normal isolated heart with eserine led to the usual sensitization to acetylcholine (Ac) at 12 min. An injection of 0.25 mg. DFP at 24 min. but little affected the sensitivity. A period of perfusion with normal Ringer-Locke fluid reduced the sensitivity of the preparation to the test dose of 0.5 μg. of acetylcholine (Ac) at 70, 77, and 120 min. Subsequent doses of 0.25 mg. at 127 min. and 1.0 mg. at 145 min. DFP markedly sensitized the heart. Time marker: 10 sec.

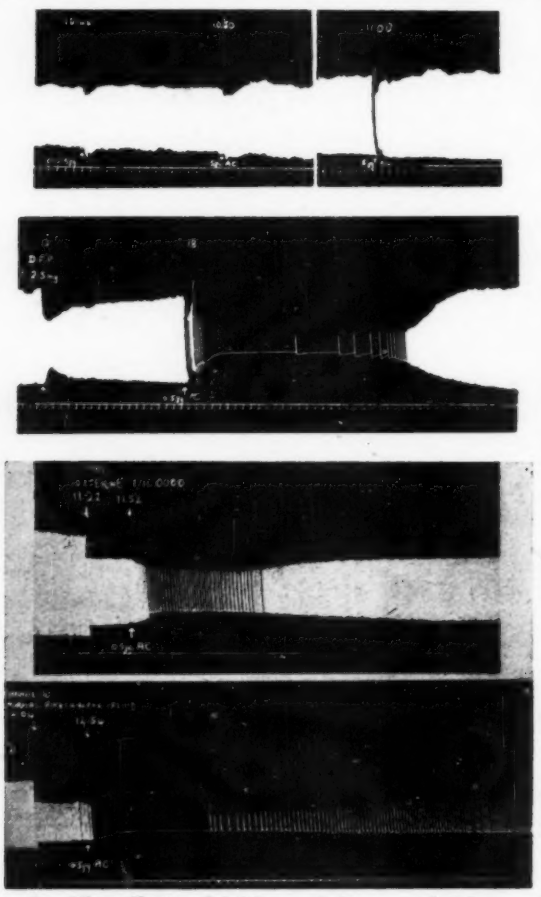

FIG. 5.—The effects of 0.05 μg., 0.5 μg. and 5.0 μg. of acetylcholine (Ac) are shown on a fresh isolated heart at 10.45, 10.50, and 11.00 respectively. A dose of 0.25 mg. DFP at 11.10 sensitized it markedly. The addition of eserine (1/100,000) at 11.22 to the perfusion fluid markedly decreased the sensitivity of the preparation to acetylcholine at 11.52, and this "atropine-like" effect was removed by continued perfusion with normal Ringer-Locke fluid as seen at 12.54.

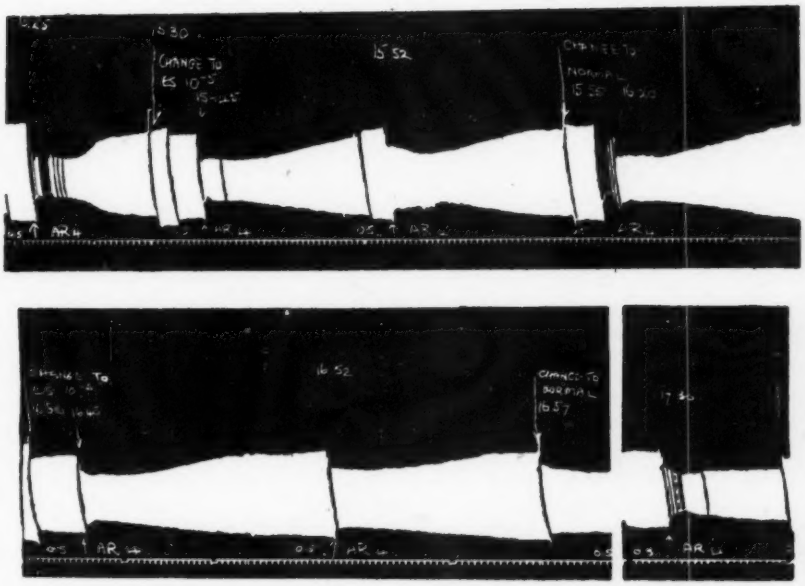

FIG. 6.—The response of the rabbit heart fully sensitized with DFP to 50 μg. arecoline (0.5AR4) is seen at 15.25. The addition of eserine (1/100,000) at 15.30 to the perfusion fluid markedly reduced the sensitivity of the preparation to the reference dose of arecoline (0.5 AR4) at 15.45 and at 15.52. When normal Ringer-Locke fluid was restored to the heart at 15.55, its sensitivity to arecoline had returned by 16.20 and the "atropine-like" effect of eserine was developed for the second time in the same preparation in the lower tracing.

(1/100,000 in Ringer-Locke solution) for a period, the depression of the heart beat produced by the reference dose of acetylcholine was greatly *reduced* compared with that produced immediately before perfusion with eserine, a change reminiscent of the effect of a small dose of atropine. If normal Ringer-Locke fluid was now restored to the heart the sensitivity of the preparation to acetylcholine returned approximately to the level obtained initially in the fully sensitized heart (Fig. 5).

A closely similar result was obtained with prostigmine: perfusion for as short a time as ten minutes with prostigmine (1/100,000 in Ringer-Locke solution) greatly reduced the sensitivity of a heart, already fully sensitized with DFP, to the reference dose of acetylcholine; then, after about an hour's perfusion with normal Ringer-Locke fluid, the sensitivity to acetylcholine returned to almost the initial post-DFP level.

If the sensitization with DFP was only partial, it was usually found that eserine or prostigmine had an additive effect, increasing sensitization already produced by a small dose of DFP. The "atropine-like" action of eserine or prostigmine was only observed in hearts which had been fully sensitized with DFP; it was, however, an unexpected finding, and the "atropine-like" action of eserine was investigated further. Normally, when the rabbit heart is fully sensitized with DFP, no change occurs in its sensitivity to pilocarpine or to arecoline. Both these drugs are powerfully antagonized by atropine in the heart, and consequently, if eserine has a true atropine-like action in a heart which has been fully sensitized with DFP, it should reduce not only the effect of acetylcholine but also the effects of pilocarpine and arecoline. Experimentally, this was found to be so, the atropine-like action of eserine being best demonstrated with arecoline (Fig. 6).

THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE

METHOD

In some experiments the method of Chang and Gaddum (1933) was employed, a 40-ml. bath being used, but in most experiments the following procedure was adopted: the muscle was suspended in a 10-ml. bath of oxygenated frog Ringer solution in such a way that it could be stimulated through two platinum electrodes fixed to each end by ligatures. The muscle was stimulated maximally by a condenser discharge and the contractions recorded on a kymograph by a tangentially writing lever. This preparation was subjected to the action of test doses of acetylcholine for precisely 60 sec. and was stimulated every 50 sec. throughout the experiment by the condenser discharge so that the effect of the various procedures upon this form of electrical stimulation could be studied. Provision was made for 5-sec. periods of faradization of the muscle.

RESULTS

When DFP was added to the bath so that the final concentration was over about 0.75 mg. per ml., a spontaneous contraction resulted from which the muscle rarely relaxed. Higher concentrations of DFP killed the tissue as indicated by its lack of response to stimuli. With lower concentrations, DFP had little immediate effect upon the muscle

beyond a slight transient depression of the contraction elicited by the condenser discharge, but after the addition of DFP to the bath the height of the contractions was very gradually and progressively enhanced (Fig. 7).

The depression of the mechanical response to motor nerve stimuli in the cat's tibialis anterior muscle observed some time after intra-arterial administration of DFP (Brown, Burns, and Feldberg, 1948) had no counterpart in the experiments with the frog's rectus. The augmentation of the mechanical response to the condensor discharge usually attained maximal dimensions in 2 to 3 hours and was well maintained over a period of several further hours.

In none of the experiments was it possible to observe fasciculation of the muscle after the addition of DFP or acetylcholine to the bath. In a few experiments the action potentials of the rectus were recorded and evidence of repetitive contraction of the muscle fibres was found in the rectus subjected to DFP (Knox and Quilliam, unpublished observation).

Changes in the sensitivity to acetylcholine

DFP markedly sensitized the frog rectus to the action of acetylcholine. About 0.1 to 0.2 mg. DFP per ml. was the optimal bath concentration. The sensitization was partially developed in 30 min. but took at least one hour to become maximal (Fig. 7). In thirty-two experiments, the increase in sensitivity to acetylcholine was quantitatively assessed and the mean value was 5 ± 3 times. On four occasions there was a tenfold and on one a thirteenfold sensitization (Fig. 8).

The type of response to acetylcholine was altered considerably. The first dose of acetylcholine after DFP caused a marked and prolonged augmentation of the muscle twitch in response to the condenser discharge stimulus (Fig. 7) lasting from 10 to 60

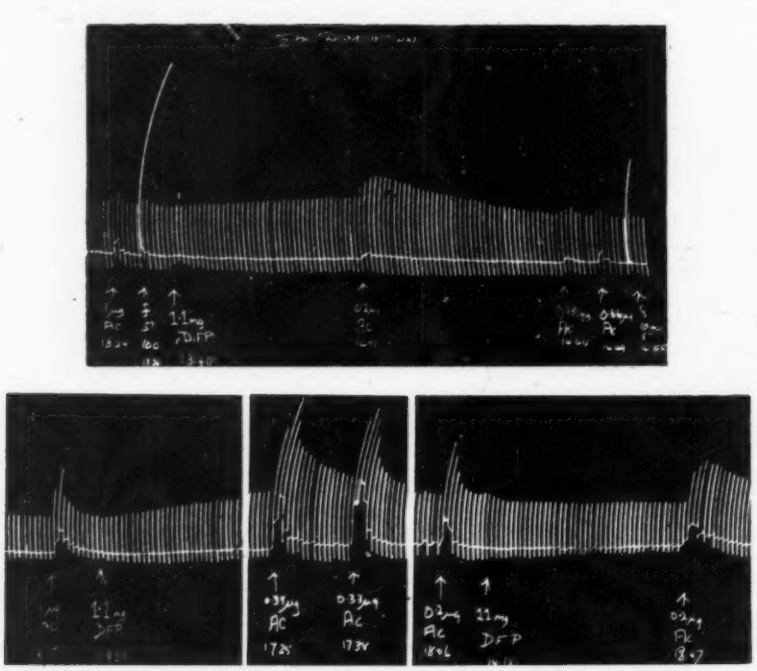

FIG. 7.—In the upper record of the isotonic contraction of the frog's rectus abdominis muscle, the effect of a reference dose of 1 μg. of acetylcholine (Ac) at 13.29 and of a 5 sec. tetanus (f 5″) at 13.34 will be seen. A slight transient depression followed by a gradual augmentation of the contraction occurred after the addition of 1.1 mg. DFP to the 10-ml. bath at 13.40. After 30 min. 0.2 μg. of acetylcholine at 14.10 demonstrates the prolonged augmentation of the contraction after the acetylcholine had been allowed to act upon the rectus for 60 sec. and then washed off three times. At 14.44 and 14.49, attempts to measure the sensitivity of the preparation showed that 0.66 μg. of acetylcholine at 14.49 just failed to give a response as great as that to 1 μg. at 13.29. At 14.55, the effects of the same short tetanus as was given at 13.34 will be seen. The post-tetanic augmentation of the contraction is seen in both instances. In a new preparation (lower tracing) exposure to DFP for 60 min. was required to develop the fivefold increase in sensitivity to acetylcholine illustrated. A second application of 1.1 mg. DFP at 18.15 caused no further increase in sensitivity. The form of the post-acetylcholine augmentation may be compared at 17.25, 17.38, 18.06, and at 18.47. The muscle was stimulated by the condenser discharge every 50 sec. The tracings are to be read from left to right.

FIG. 8.—Frog's rectus abdominis. Method of Chang and Gaddum (1933); 40-ml. bath; a thirteenfold sensitization of a frog's rectus will be seen after treatment with 25 mg. DFP for 76 min.

minutes or more, even after several washings. This post-acetylcholine augmentation was seen after subsequent doses of acetylcholine but only to a smaller degree. After prolonged exposure to DFP the augmentation after the first dose of acetylcholine was less marked.

The sensitivity to acetylcholine in control muscles showed a slight decline with time and there was also a similar slight falling off in the sensitivities of DFP-treated muscles of about the same order. The sensitization with which DFP endowed the muscle appeared to last as long as 6–8 hours without any but the slight reduction comparable with that seen in control muscles.

Changes in response to faradic stimulation

In the normal rectus, a 5-sec. period of faradization resulted in a powerful, brief contraction, and several succeeding muscle twitches in response to the condenser discharge were larger than normal as found by Brown and von Euler (1938) in mammalian muscle (Fig. 7). After a period of exposure to DFP, the muscle responded to faradic stimulation by a contracture lasting for some minutes, upon which the muscle twitches in response to the condenser discharge were superimposed (Fig. 9). Feng (1936) observed a similar contracture in the toad's sartorius muscle on faradic stimulation of the nerve after eserine.

Action of eserine after DFP

In view of the " atropine-like " action that eserine can exert in the rabbit's heart after full sensitization with DFP, it was of interest to see whether a similar action could be demonstrated in the frog's rectus muscle. Fig. 9 illustrates a typical experiment: a rectus abdominis muscle, already fully sensitized with DFP, was exposed to eserine (1/100,000) and

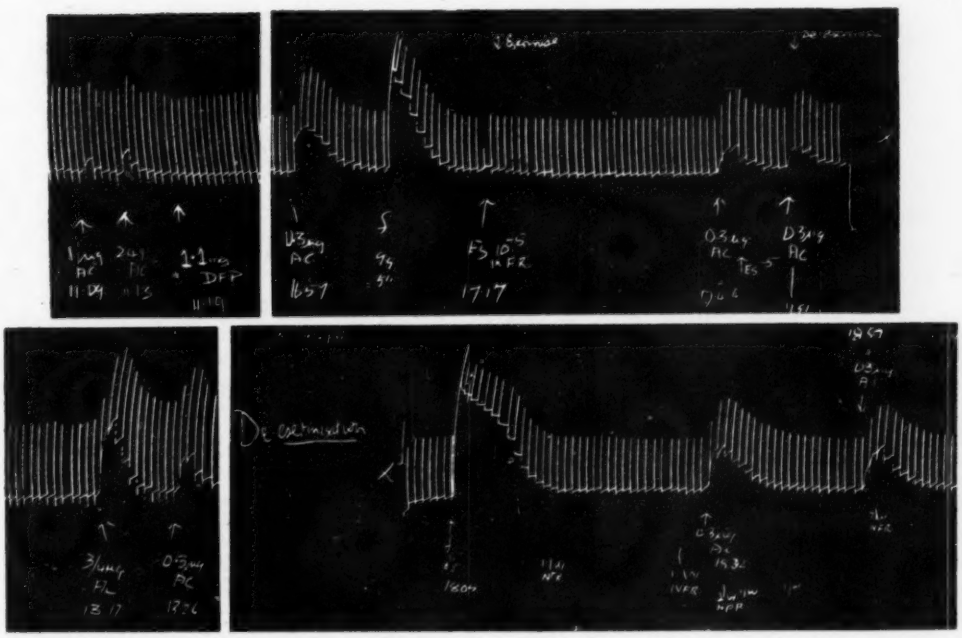

FIG. 9.—The upper left tracing shows the sensitivity of a rectus to 1 μg. and 2 μg. of acetylcholine (Ac) at 11.09 and 11.13 respectively. At 11.19 1.1 mg. DFP was added to the 10-ml. bath, and after 118 min. the increase in sensitivity was over sevenfold as indicated by 0.3 μg. of acetylcholine at 13.26 in the lower left tracing. In the upper right tracing 3½ hours later, the sensitivity of the rectus is well maintained as the action of 0.3 μg. of acetylcholine at 16.57 indicates. Next at f, the contracture following a standard period of tetanization is seen. At 17.17 eserine (1/100,000) was added to the bath and the sensitivity to the test dose, 0.3 μg. acetylcholine, progressively diminished at 17.44 and at 17.51. In the lower right tracing washing of the preparation was commenced at 17.54 and at 18.05 the effect of a standard tetanization is seen, and at 18.32 and 18.50 the sensitivity of the preparation to the test dose of acetylcholine has returned.

27 minutes later a test dose of acetylcholine produced a smaller effect than it had done before eserine. This action of eserine can be removed by washing the preparation repeatedly with normal frog Ringer fluid. Brown (1937) showed that atropine injected intra-arterially into a frog's gastrocnemius muscle depressed the response of the muscle to acetylcholine similarly injected.

Action of DFP on the muscle after eserine

The addition of DFP to the bath when the rectus had been maximally sensitized with eserine resulted in no further increase in the sensitivity of the preparation to acetylcholine.

The actions of curare and atropine

If a sufficiently high concentration of either of these two substances is added to the bath it is possible to block the action of acetylcholine upon the rectus either completely or nearly so. The response of the muscle to the condenser discharge stimulus is unaltered. The blocking action of curare can be removed by soaking the muscle in Ringer solution containing DFP for a long time. DFP can also reverse to some extent the effects of atropine upon the frog rectus.

The action of propylene glycol upon the rectus abdominis muscle

One muscle of a pair of recti was set up in a series of experiments as a control and received the same quantity of propylene glycol as that given in a dose of DFP to the test preparation. With the quantities of propylene glycol used in our experiments with DFP, no changes in sensitivity of the rectus were seen.

As propylene glycol is often used as a solvent for drugs, it was of interest to observe the effect of larger doses upon the rectus. When 1.5 ml. or more were added to the 40-ml. bath for 40 minutes there was a slight increase in sensitivity of the preparation to acetylcholine. In addition three other phenomena were seen. A series of large spontaneous contractions might develop which were sometimes rhythmical in nature and which might give place to small spontaneous twitches. Both these reactions could be precipitated by washing but often occurred without. The large rhythmical contractions usually lasted for a few minutes, but the twitching lasted from one half to one hour; repeated washing of the preparation appeared to accelerate the disappearance of both phenomena. The third change seen was an exaggerated stretch response. Normally an isolated frog rectus does not respond to a slight stretch, but in a preparation which has received an excess of propylene glycol a slight mechanical stretch results in one or more large spontaneous contractions. This exaggerated stretch response was associated with the period during which the twitches were seen and was, like the slight increase in sensitivity to acetylcholine, removed by repeated washings. Smaller quantities of ethylene glycol produced similar changes.

Discussion

The sensitization to acetylcholine with which DFP endows the isolated rabbit's heart differs from that seen after eserine or prostigmine in the following manner: a single injection of 0.25 mg. of DFP can produce a permanent one-hundredfold sensitization within two minutes. Eserine or prostigmine must be added to the perfusion fluid for at least 10 to 20 minutes in order to sensitize the heart to acetylcholine, and they are both easily removed by continued perfusion with normal Ringer-Locke fluid.

The resistance to the action of DFP that the heart, fully sensitized with eserine or prostigmine, exhibits is probably due to saturation of the cholinesterase of the tissue with eserine or prostigmine; when the eserine or prostigmine is removed—e.g., by perfusion with normal fluid—the cholinesterase is free to combine irreversibly with DFP and the tissue becomes permanently sensitized to acetylcholine.

It is of interest to note that a patient who has been receiving prostigmine regularly in the course of treatment for myasthenia gravis does not respond so satisfactorily to DFP as an untreated patient. This is a clinical counterpart to the experimental observation that prostigmine can protect tissues against DFP. Koster (1946) has shown that eserine can exert some protective action in cats against lethal doses of DFP.

The blocking effect that eserine exerted on the actions of acetylcholine, pilocarpine, and arecoline in a heart already fully sensitized with DFP was unexpected and, so far as could be determined, was "atropine-like" in nature; the acetylcholine sensitivity of the fully sensitized frog's rectus could also be reduced with eserine. These actions of eserine are quite distinct from its anticholinesterase activity and are only unmasked when the cholinesterase activity of the tissue has been fully inhibited. In each preparation the action was abolished by continued washing of the preparation in such a way as to remove eserine.

In the frog's rectus abdominis muscle DFP was found to be a more reliable and more effective sensitizing agent to acetylcholine than eserine; it had a permanent action but required at least one

hour for its effect to be fully exerted. Eserine, however, can sensitize the rectus in about 20 min., and a dose of eserine about one-tenth that of DFP was required.

Miquel (1946) stated that eserine increased the acetylcholine sensitivity of the frog's rectus after DFP. In our work we found that the quantities of DFP used by Miquel were insufficient to sensitize the rectus fully. In these circumstances a dose of another anticholinesterase drug would be expected to enhance the sensitivity of the tissue still further. But when the tissue is fully sensitized with DFP and exposure to more DFP does not alter the sensitivity, the administration of eserine results in a decrease in sensitivity and not an increase as found by Miquel.

Bacq's (1947) findings were also different from those of Miquel; he found that eserine did not change the acetylcholine sensitivity of the frog's rectus after DFP. In our experience, it is only by a study of the mechanical response of the rectus to electrical stimulation that the depressant action of eserine after full sensitization with DFP can be demonstrated.

Finerty (1947) showed that some potentiation of the acetylcholine response of the isolated rectus can be obtained with phosphoric acid, and he considered that the acidic properties of a solution of DFP that has deteriorated are in part responsible for the sensitization seen after this drug. However, the phosphoric acid sensitization illustrated was of the same order as that which we found after excessive doses of propylene glycol and was probably non-specific in nature. In addition, the periods of exposure to DFP used by Finerty seem extremely brief.

One of the most striking features of the action of DFP upon the rectus is the change in form of the response to the test dose of acetylcholine. The augmentation of the mechanical response to the condenser discharge stimulus was maintained for as long as sixty minutes even after three washings. More repeated washing encouraged the phenomenon to disappear. Brown (1937) considered that "in general, the reactions of normally innervated frog (*gastrocnemius*) muscle closely resembled those of mammalian muscle after denervation" rather than those of normally innervated mammalian muscle. Brown, Burns, and Feldberg (1948) found that in the cat's tibialis anterior muscle, whether denervated or not, the action of acetylcholine after DFP was to depress the mechanical response to stimulation. We saw no counterpart of this reaction in the rectus, but Coppée and Bacq (1947), using the frog's sciatic nerve-gastrocnemius preparation, illustrate such a depression both after a period of brief tetanic stimulation and after acetylcholine in the normally innervated frog's muscle after exposure to DFP.

Summary

1. The actions of DFP upon the perfused isolated rabbit's heart and the isolated frog's rectus abdominis muscle preparations are described.

2. A single injection of 0.25 mg. DFP will endow the isolated heart with a permanent hundredfold sensitization to acetylcholine which is fully developed within two minutes.

3. Whereas an injection of DFP does not alter the acetylcholine sensitivity of a heart already fully sensitized with eserine or prostigmine, the addition of eserine or prostigmine to the perfusion fluid of a heart fully sensitized to acetylcholine by DFP results in a *reduction* in its sensitivity; restoration of the normal perfusion fluid for a period causes the sensitivity of the heart to return. This "atropine-like" action of eserine is also seen with pilocarpine and arecoline in hearts that have previously received DFP.

4. After soaking the frog's rectus in a solution of DFP for at least one hour, its acetylcholine sensitivity may be enhanced permanently 5 ± 3 times. The response of such a sensitized rectus to maximal condenser discharge stimuli after acetylcholine is markedly augmented, often for quite a considerable period. The application of DFP to the rectus is not followed by depression of its response to electrical stimuli at any phase.

5. Administration of eserine to a rectus, already fully sensitized to acetylcholine with DFP, leads to a reduction in its sensitivity which can be restored to normal by repeated washing with normal frog Ringer solution.

6. In the amounts used in experiments with DFP, the solvent propylene glycol exerts no effect upon the acetylcholine sensitivity of the isolated rabbit's heart or the frog's rectus. When large quantities of propylene glycol are added to the bath containing a frog's rectus the following changes may occur: (*a*) a slight increase in the acetylcholine sensitivity, (*b*) large spontaneous twitches which may be rhythmical, (*c*) small spontaneous non-rhythmical twitches, and (*d*) an exaggerated stretch response. All these changes are abolished by repeated washing.

7. These pharmacological actions of DFP are discussed with special reference to eserine and prostigmine. Certain differences in the response of

the rectus to DFP when compared with mammalian and amphibian nerve muscle preparations are indicated.

It is a pleasure to thank Prof. R. J. S. MacDowall and Dr. D. B. Taylor for advice.

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THE TOXICITY OF TRICHLORETHYLENE

BY

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(Received February 11, 1949)

Before it came to be used for anaesthetic purposes, trichlorethylene was recognized as one of the safest fat solvents available for industrial use (M.R.C. Report 80, 1937; Hamilton, 1934), and to-day, eight years after the widespread adoption of this drug as a general anaesthetic, the literature contains only two cases of acute yellow atrophy which have been laid to its charge (Herdman, 1945; *Lancet*, 1944). Further investigation of the toxicity of trichlorethylene in the quantities used for anaesthesia has been carried out in the human subject, using the cephalin flocculation test as a measure of liver function (Armstrong, 1947). The results of this study showed that trilene produced less disturbance of liver function than the classical and relatively safe anaesthetic agent, ether. None the less, trichlorethylene is a close chemical relation of the known hepatotoxins chloroform, carbon tetrachloride, and tetrachlorethane, and there has been for long a lingering suspicion that, if enough of this agent were given, liver necrosis might ensue. In fact, in a study of the anthelmintic action of trichlorethylene and other drugs, Barsoum and Saad (1934) state that this, in common with the other agents of the same chemical group, will produce fatty degeneration of the liver when given by mouth to experimental animals. This may, of course, temporarily involve high concentrations in the portal blood and liver. It therefore seemed desirable to carry out a further investigation on the action of trichlorethylene on the liver.

Many experimental studies of the toxicity of trichlorethylene have already been reported, but most of these were directed to the discovery of the dangers of the use of the drug in industry. Accidental narcosis to the point of respiratory arrest is the commonest cause of death in industrial accidents. Several workers (Lehmann, 1911; Lehmann and Schmidt-Kehl, 1936) therefore rate the toxicity of chlorinated hydrocarbons in the order of their anaesthetic potency, and regard trichlorethylene as dangerous because it is a powerful agent in low concentrations. Other studies (Lande *et al.*, 1939) were more concerned with the chronic toxicity of the drug, but only in the amounts in which it might be inhaled by those using it in the course of their work. The only workers who carried the administration of trichlorethylene to the point of anaesthesia on a series of occasions were Krantz and his colleagues (1935). Once anaesthesia had been induced in their animals, however, they made no attempt to maintain it, nor was the concentration of the drug measured in this study. It was the purpose of the present investigation to submit animals repeatedly for as long as possible to the maximum amount of trichlorethylene vapour they would tolerate.

METHODS

For the purposes of this experiment, it was necessary to design an apparatus which would provide a vapour of constant strength for a considerable period. An ordinary Boyle's apparatus with a trilene bottle obviously would not serve. It is well known that the amount of anaesthetic vaporized by the gases in such a machine depends on the rate of flow, the force with which the gases impinge on the liquid and the temperature of the liquid. Only the first of these variables is controlled in a Boyle's apparatus. As the liquid in the bottle evaporates, its level falls and the force with which the gases impinge on the liquid is reduced. Similarly, because the temperature of the liquid falls as evaporation occurs, the partial pressure of the liquid becomes less and a smaller amount is volatilized by the gaseous stream. It proved difficult to obtain a satisfactory thermostatically controlled heater to place in the trilene bottle, nor did it seem easy to make an adequate apparatus for main-

taining the level of the liquid in the bottle at a constant height. In the earliest experiments, therefore, a known quantity of trichlorethylene was vaporized in a large aspirator of known capacity, the contents of which could be mixed by a small fan. A measured quantity of the liquid was run into a piece of cotton-wool attached to the blades of the fan. As soon as these began to spin, the trichlorethylene was thrown by centrifugal force on to the walls of the aspirator, where it was almost immediately vaporized by the air stream. After the liquid had been vaporized, the fan was not run continuously, but was turned on for five minutes in each quarter-hour. The amount of oxygen taken up and the amount of carbon dioxide produced by 10 mice in 1 hour was insufficient to vitiate the results with an aspirator bottle of 11.5 litres capacity. Later, however, it became necessary to provide a steady flow of gas containing a known amount of trichlorethylene. For this purpose, a pump running at a constant speed with a displacement of about 16 litres a minute was used. It passed air over trichlorethylene in a 250 c.c. conical flask partially immersed in a water bath whose temperature was thermostatically controlled. After about 20 minutes' running a steady temperature gradient was established between the trichlorethylene and the water, and a vapour of constant strength was delivered. Further, only an initial calibration was necessary and thereafter the temperature of the trichlorethylene was an accurate indication of its concentration in the mixture. The rate of evaporation with this apparatus was so slow that only a negligible change in the level of the liquid occurred during one hour's use. To make it easy to obtain mixtures containing small amounts of trichlorethylene vapour, a by-pass for part of the air was added.

In order to calibrate this apparatus, it was necessary to find some accurate method of estimating small quantities of trichlorethylene in air. The Fujiwara reaction, as described by Habgood and Powell (1945), was used, but the results were found to be too readily influenced by minor variations in analytical technique. In addition, considerable difficulty was found in bringing the alkali into contact with the trichlorethylene dissolved in the supernatant toluene layer. An attempt was also made to use Waller's chloroform balance (Macintosh and Mushin, 1946), but it, too, was not sufficiently accurate for the purposes of the experiment. In the end a method originally designed by Dr. E. Falkner Hill for the estimation of chloroform was employed.

The basis of this technique is the direct measurement of the increase in weight which occurs when the air in a flask is replaced by a more dense mixture of trichlorethylene and air. The author found it necessary to make certain modifications in the original technique. The error which was due to difference in temperature between the air outside the flask and the gas within it was eliminated by passing the mixture containing trichlorethylene through a long cooling coil immersed in a large volume of water at room temperature. The calculation, too, was modified to correct for the weight of the air displaced from the flask by the trichlorethylene. The details of the method are as follows:

The special flask was weighed containing ordinary air at room temperature. Air containing trichlorethylene was then blown through it until its weight became constant. After a trial, it was found that five minutes was enough for this purpose. The flask was then weighed again and finally its capacity determined by filling it with water. The details of the calculation, which is based on elementary conceptions of specific gravity, will be found in the Appendix.

Animal Experiments

In the first experiment a batch of 10 mice was exposed for one hour to 1 per cent trichlorethylene vapour in air daily, except on Sundays, for 12 days. Three animals died during the first inhalation of the mixture, but the remainder survived except that during each of the ninth, eleventh, and twelfth exposures one mouse died. During the earlier inhalations, however, animals were sometimes removed from the vapour before the whole hour had elapsed, when they were obviously on the point of respiratory arrest. The next experiment was exactly similar except that 1.5 per cent trichlorethylene was administered and no attempt was made to rescue animals on the point of death until the whole hour had passed. With this concentration, the toxicity of the drug was even more marked and none of the animals survived the eighth inhalation.

Post-mortem examination of these animals revealed no direct evidence of the cause of their deaths, nor did it afford any explanation why those which survived one hour's exposure to trichlorethylene on one day should succumb after exactly the same treatment on the day following. It seemed fairly clear that the immediate cause of the deaths was anoxia. Thus the animals which were not going to survive became cyanotic during the inhalation and ultimately developed gasping respirations which became progressively less frequent until the animal died. A few had typical epileptiform convulsions which were also evidence of anoxia. Finally, a proportion of the animals whose breathing had ceased was resuscitated by artificial respiration. The mechanism of the production of the anoxia was fairly clear. As in the human subject, so in these mice, trichlorethylene caused tachypnoea. Because the drug augments the sensitivity of the pulmonary stretch receptors (Whitteridge and Bülbring, 1946), this increase in respiratory rate occurred at the expense of the depth of the breathing. Presently, the respiratory excursion became so small that the air drawn in probably filled little more than the dead space, and alveolar ventilation was reduced to negligible proportions, with consequent development of progressively increasing anoxaemia.

In view of the facts set out in the last paragraph, it seemed probable that mice would withstand anaesthesia with trichlorethylene very much better in oxygen than in air. To test this thesis, a batch of 10 mice was exposed for one hour as before to 1.2 per cent trichlorethylene vapour in oxygen. At the end of the first inhalation, all the animals were well, but the laboratory attendant found two of them dead in their cage half an hour later. It seemed probable that these animals had suffered asphyxia because they were suddenly removed from an atmosphere rich in oxygen while their respiration was still too shallow to maintain life when they were breathing air. At the end of each inhalation thereafter, therefore, the animals were gradually weaned over a period of about five minutes from oxygen to air. There was no further mortality until the seventh inhalation was about to begin, when two mice were found dead in the cage. A third animal died during the succeeding night. All these animals were found to be suffering from pneumonia at post mortem, and it is, of course, well known that small animals frequently exposed to high concentrations of oxygen may develop this condition. For this reason the experiment was not continued, but it was felt that a sufficient number of animals had survived a large enough dose of trichlorethylene often enough to make it clear that the drug was less toxic when given in oxygen than in air.

As has already been indicated, post-mortem examination of the animals which died during exposure to trichlorethylene revealed no immediate cause of death. Two animals were apparently suffering from subacute nephritis of relatively long standing, but the kidneys of the remainder and the livers of all the animals were healthy on naked-eye examination. Sections were made of the livers and kidneys of the animals, and, apart from the nephritis already noted on naked-eye examination, there was no evidence of tissue destruction in either of these organs which might have been the result of the action of trichlorethylene. There was some disappearance of liver glycogen in the animals which had died during anaesthesia, but in the four which survived the first series of inhalations and were sacrificed later the livers and kidneys were indistinguishable from those of healthy mice. In order to obtain further information on this matter, an additional series of six mice was exposed to 1 per cent trichlorethylene for forty minutes on twelve occasions and their livers and kidneys submitted to a pathologist for examination. Again it was reported that there were no changes which could be ascribed to the toxic action of trichlorethylene on these organs. Finally, in order to make quite certain that the animals used were relatively sensitive to hepatotoxins, a series of ten mice was exposed to 1.0 per cent chloroform on twelve successive occasions. Five died of overdose during the first inhalation, which lasted an hour. On successive days, the remainder were exposed for forty minutes. Two died, one during the third and one during the seventh inhalation, and, at post mortem, both showed the shrunken, fatty liver typical of delayed chloroform poisoning.

Summary

The results of this investigation indicate that trichlorethylene is a potential poison to mice. Further, their sensitivity to the drug seems to increase with repeated exposure. Animals under the influence of trichlorethylene appear to die because the drug increases the rate of their respirations until ventilation becomes inefficient. In the doses used in this study, the agent had no toxic action on the liver or kidneys. The practical importance of the findings of this investigation to clinical anaesthesia are twofold. First, they emphasize the desirability of giving plenty of oxygen to patients who develop tachypnoea as a response to trichlorethylene. Secondly, they confirm the previously made observations that this drug has no serious toxic effects on the parenchyma of the liver and kidneys of healthy mice, even when administered repeatedly in anaesthetic concentrations for an hour.

I am indebted to Professor A. D. Macdonald for much valuable advice and encouragement.

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Appendix

The details of the calculation for the estimation of trichlorethylene by the method used in this study are:

Let W_1 be the result of the first weighing.
W_2 be the result of the second weighing.

By the principle of Archimedes,

W_1 and W_2 are the absolute weights of the flask and its contents less the weight of air displaced by the flask.

$W_2 - W_1$ = weight of gaseous mixture in the flask − weight of air which the flask could contain.

or Weight of trichlorethylene vapour in the flask + weight of air still in flask − weight of air which the flask could contain.

or Weight of the trichlorethylene vapour − the weight of the air which it has displaced from within the flask. (1)

These two quantities have the same volume,

$$\therefore \frac{\text{Wt. of air displaced by trichlorethylene}}{\text{Density of air}} = \frac{\text{Wt. of trichlorethylene}}{\text{Density of trichlorethylene}}$$

$$\text{i.e., Wt. of air displaced by trichlorethylene} = \frac{\text{Weight of trichlorethylene vapour}}{\text{Density of trichlorethylene vapour}} \times \text{Density of air}$$

Substitute this expression in (1), using symbols D_T and D_A for densities of trichlorethylene and air respectively.

$W_2 - W_1$ = weight of trichlorethylene vapour in the flask

$$- (\text{weight of trichlorethylene in flask}) \times \frac{D_A}{D_T}$$

$$= \text{weight of trichlorethylene in flask} \left(1 - \frac{D_A}{D_T}\right)$$

$$\therefore \text{Weight of trichlorethylene in flask} = \frac{W_2 - W_1}{\left(1 - \frac{D_A}{D_T}\right)}$$

Now 1 gram molecule of any gas at 0° C. occupies 22.4 litres, approximately.

The molecular weight of trichlorethylene is 131.5.

$$\text{Volume of trichlorethylene in flask} = \frac{\text{Weight of trichlorethylene}}{131.5} \times 22.4 \text{ litres}$$

$$= \frac{22.4(W_2 - W_1)}{131.5\left(1 - \frac{D_A}{D_T}\right)} \text{ at } 0° \text{ C.}$$

If the determination is carried out at room temperature of t° C., the volume obtained from the last equation will require to be corrected by multiplication by $\frac{t + 273}{273}$ (Charles's Law).

Brit. J. Pharmacol. (1949), **4**, 181.

THE ENZYMIC HYDROLYSIS OF TRIACETIN (GLYCERYL TRIACETATE)*

BY

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(Received February 11, 1949)

The ability of cobra venom to hydrolyse acetylcholine was first described by Jynegar, Sehra, Mukerji, and Chopra (1938). The esterase responsible for this reaction was further studied by Bovet and Bovet (1943) and Bovet-Nitti (1947); they established that the hydrolysis was brought about by an enzyme distinct from the lecithinase present. Bovet-Nitti (1947) showed that the venom was able to hydrolyse a number of aliphatic esters, in particular acetyl esters including triacetin, and she therefore considered that the enzyme responsible was not a cholinesterase but an "acetylase" which would hydrolyse esters of acetic acid including acetylcholine.

Zeller (1947) found that the cobra venom esterase would hydrolyse acetyl-β-methylcholine but not benzoylcholine, and he classified it as a "true" cholinesterase according to the classification of Mendel and Rudney (1943). In later publications Zeller (1948a and b), in agreement with Bovet-Nitti (1947), sees in the ability to hydrolyse aliphatic esters a property which distinguishes the cobra venom enzyme from the mammalian true cholinesterase.

At the same time, however, Bodansky (1946) had found that triacetin was hydrolysed by mammalian tissues. The hydrolysis by mouse brain was inhibited by DFP (di*iso*propylfluorophosphonate). He obtained preparations of cholinesterase from red cells which hydrolysed acetylcholine and triacetin but not methyl butyrate and tributyrin.

These findings suggested the possibility that the pattern of substrate specificity of the cobra venom enzyme was in fact similar to that of the true cholinesterase of mammalian tissues, and in this paper we have therefore investigated whether triacetin and choline esters are hydrolysed by the same enzyme. Three preparations were studied: cobra venom, horse serum, and a homogenate of dog's caudate nucleus.

* Communicated in part to the Biochemical Society (Holton, 1948).

Methods and Materials

1. *Substrates*

Acetylcholine bromide and acetyl-β-methylcholine chloride were commercial samples. Benzoylcholine chloride was prepared by Dr. H. R. Ing. Triacetin was a commercial sample redistilled, suspended in Ringer's solution, and brought to *p*H 7.4 before use.

2. *Enzyme preparations*

Cobra venom was given to us by Dr. C. H. Kellaway, F.R.S. It was dissolved in Ringer's solution. Horse serum was sent by Dr. E. S. Duthie from the Lister Institute, Elstree; a purified preparation of horse serum cholinesterase, free of ali-esterase, prepared according to Strelitz (1944) was kindly sent by Dr. J. W. Legge. A homogenate of dog's caudate nucleus was prepared and stored as a suspension in Ringer's solution (100 mg. fresh weight of tissue per ml.).

For the measurement of enzymic activity manometric methods were used. In all experiments the temperature was 37.5° C. and the reaction was carried out in an atmosphere of 95 per cent N_2 and 5 per cent CO_2 in Krebs's bicarbonate Ringer. Unless otherwise stated the reaction volume was 3.0 ml. The small blank due to non-enzymic hydrolysis is subtracted in the results given below.

Results

1. *Horse serum* (Table I)

It is known that the cholinesterase of horse serum will hydrolyse benzoylcholine (Mendel, Mundell, and Rudney, 1943). Fig. 1*b* shows the rate of hydrolysis of 0.006*M* benzoylcholine and of 0.006*M* triacetin and also of a mixture of the two esters. At a concentration of 0.006*M* benzoylcholine the horse serum cholinesterase is known to be saturated with substrate (Mendel, Mundell, and Rudney, 1943). It will also be seen that the rate of liberation of CO_2 with the mixture is equal to the sum of the rates with the esters separately. This indicates that triacetin is hydrolysed by an enzyme other than cholinesterase.

This result was confirmed by an experiment with the purified preparation of horse serum cholinesterase of which 0.5 ml. was used per flask. The result given in Table I shows that both acetylcholine and benzoylcholine were actively hydrolysed but that the hydrolysis of triacetin was negligible compared with its hydrolysis by untreated horse serum.

TABLE I

HYDROLYSIS OF CHOLINE ESTERS AND OF TRIACETIN IN HORSE SERUM

Substrate concentrations 0.006*M*

Substrates	μl. CO_2 liberated in 15 min. with	
	0.2 ml. horse serum	purified horse serum cholinesterase
Acetylcholine (ACh) ..	—	29
Benzoylcholine (BzCh)	62	20
Triacetin (TA) ..	46	1
BzCh + TA	108	—

The cholinesterase of horse serum is known to be inhibited by Nu 683 [(2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide dimethylcarbamate] (Hawkins and Gunter, 1946). Table II shows that the hydrolysis of triacetin was unaffected by concentrations of Nu 683 which completely inhibited the hydrolysis of benzoylcholine.

TABLE II

THE EFFECT OF Nu 683 ON THE ESTER HYDROLYSIS IN HORSE SERUM

Substrate	μl. CO_2 liberated in 15 min. in presence of increasing concentrations of Nu 683				
	0	$10^{-10}M$	$10^{-9}M$	$10^{-8}M$	$10^{-7}M$
0.006*M* BzCh	60	56	54	13	0
0.006*M* TA	150	140	140	146	142

From these experiments it can be concluded that the very active hydrolysis of triacetin by horse serum is due to an enzyme other than cholinesterase ; a very feeble hydrolysis by the cholinesterase cannot be excluded. There is known to be an ali-esterase in horse serum which hydrolyses tributyrin and methyl butyrate (Easson and Stedman, 1937 ; Richter and Croft, 1942), and it is possible that this enzyme is responsible for the hydrolysis of triacetin.

2. *Cobra venom*

Competition experiments were carried out using 0.25 mg. cobra venom. The result of such an experiment is shown in Fig. 1*c*. With cobra venom the rate of the reaction with the two esters is intermediate between the rates with acetylcholine and triacetin separately.

The action of inhibitors.—Two inhibitors of cholinesterases were used : eserine salicylate and

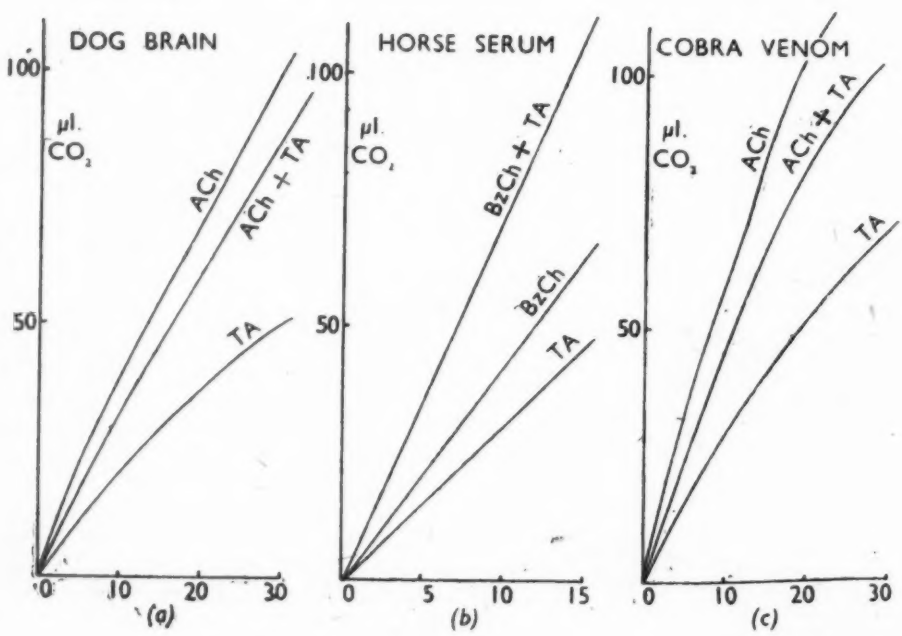

FIG. 1.—Competition experiments : (*a*) dog's caudate nucleus ; (*b*) horse serum ; (*c*) cobra venom. Abscissae : time in minutes. Ordinates : μl. CO_2 liberated.

Nu 683. The substrate concentrations were so chosen that the enzyme was approximately saturated. The results of these experiments are shown in Table III. For acetylcholine and triacetin the percentage inhibitions obtained were similar.

TABLE III

ESTER HYDROLYSIS BY COBRA VENOM IN THE PRESENCE OF INHIBITORS (Nu 683 AND ESERINE)

Inhibitor	Substrate	Percentage inhibitions during first 15 min. with inhibitor concentrations:			
		$10^{-8}M$	$10^{-7}M$	$10^{-6}M$	$10^{-5}M$
Nu 683	0.005*M* ACh	34	75	83	98
	0.03*M* TA	34	81	93	98
Eserine	0.005*M* ACh	26	66	80	92
	0.03*M* TA	20	74	92	96

These results are in agreement with the views of Bovet-Nitti (1947), according to whom the same enzyme is responsible for the hydrolysis of acetylcholine and triacetin.

3. *Dog's caudate nucleus*

The competition experiment illustrated in Fig. 1*a* shows that the hydrolysis of a mixture of 0.006*M* acetylcholine and 0.03*M* triacetin was slower than the hydrolysis of acetylcholine alone. A similar result was obtained when a mixture of triacetin and acetyl-β-methylcholine was used. The amounts of CO_2 evolved in 15 min. were:

from 0.03*M* acetyl-β-methylcholine	24 μl.
from 0.03*M* triacetin	36 μl.
from both esters	31 μl.

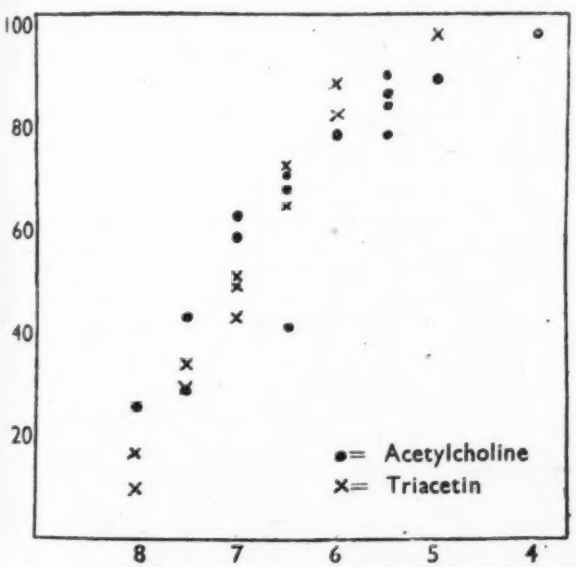

FIG. 2.—Inhibition by eserine of enzymic hydrolysis of acetylcholine (dots) and triacetin (crosses) by a suspension of dog's caudate nucleus. Each flask contained the equivalent of 4 mg. of fresh weight of tissue. Abscissa: negative logarithm of molar concentration of eserine. Ordinate: per cent inhibition.

The action of inhibitors.—In these experiments both substrates were used in concentrations which were near saturation concentration. If the esters were hydrolysed by the same enzyme it would be expected that the percentage inhibitions found under those conditions for any given inhibitor would be the same. The concentrations used were 0.006*M* acetylcholine and 0.03*M* triacetin. The results obtained with eserine salicylate are given in detail in Fig. 2, which shows that the percentage inhibitions with both substrates were not significantly different.

TABLE IV

ESTER HYDROLYSIS BY DOG'S CAUDATE NUCLEUS IN THE PRESENCE OF INHIBITORS

Inhibitor	Molar concentration	Percentage inhibition in first 15 min. with substrates	
		0.006*M* ACh	0.03*M* TA
Eserine ..	10^{-8}	13	14
,, ..	10^{-7}	62	49
,, ..	10^{-6}	80	87
,, ..	10^{-5}	90	100
Nu 683 ..	10^{-8}	4	2
,, ..	10^{-7}	40	22
,, ..	10^{-6}	78	64
,, ..	10^{-5}	88	84
Caffeine ..	10^{-3}	32	64
Benzoylcholine	10^{-2}	32	72
,,	3×10^{-2}	56	76
BQ10* ..	10^{-8}	23	14
,, ..	10^{-7}	52	46
,, ..	10^{-6}	90	72
,, ..	10^{-5}	100	76

* Decamethylenebisquinolinium dibromide

Similar results were obtained with other inhibitors in experiments summarized in Table IV. The substance BQ10 is decamethylenebisquinolinium dibromide recently prepared by Barlow and Ing (1948). Caffeine was shown to be an inhibitor of brain cholinesterase by Zeller and Bissegger (1943) and by Nachmansohn and Schneemann (1945).

These experiments suggest that the cholinesterase of dog's caudate nucleus hydrolyses triacetin.

Discussion

The experiments described in this paper are in agreement with the assumption that the cholinesterases present in cobra venom and dog's caudate nucleus are responsible for the hydrolysis of triacetin. The caudate nucleus is a tissue particularly rich in the "true" cholinesterase of Mendel and Rudney (1943). They define this enzyme as one "acting exclusively on choline esters." Our results show that this definition will have to be modified, and that the specificity towards acetylcholine is only relative and not absolute, as already postulated by Nachmansohn (1946).

Our experiments with caudate nucleus are in agreement with similar experiments of Adams and Whittaker (1948) on a purified preparation of "true" cholinesterase from human erythrocytes in which the hydrolysis of triacetin was found to be catalysed by the cholinesterase present. They found that this enzyme will split many other aliphatic esters besides acetylcholine. It thus appears that the ability of cobra venom to hydrolyse esters of acetic acid other than acetylcholine does not distinguish it from the "true" cholinesterases of mammalian tissues, and hence it seems unnecessary to classify the cobra venom enzyme as a third type of cholinesterase ("C" type of Zeller, 1948a, b).

Summary

1. The hydrolysis of triacetin by three sources of cholinesterase—horse serum, cobra venom, and dog's caudate nucleus—was investigated.

2. Triacetin hydrolysis by horse serum is mainly due to an enzyme other than cholinesterase.

3. The cholinesterases of cobra venom and dog's caudate nucleus hydrolyse triacetin.

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Brit. J. Pharmacol. (1949), **4**, 185.

THE ASSAY OF ANTICHOLINESTERASE DRUGS BY THE CHROMODACRYORRHOEA RESPONSE IN RATS

BY

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(Received February 12, 1949)

With the introduction of new anticholinesterase compounds of the alkyl phosphate group an accurate assessment of their potencies and duration of action relative to such well-established drugs as eserine and neostigmine has become important. It was quickly found that attempts to assess them pharmacologically by various isolated organ preparations were impracticable because the alkyl phosphates act much more slowly than eserine and neostigmine and their effects are irreversible during the time of an assay. It was thought that an assay method in an unanaesthetized animal would overcome many of these difficulties and yield data of more direct value.

The phenomenon of chromodacryorrhoea in rats, first described by Freud (1933), seemed to be a promising basis for a test, if it could be made satisfactorily quantitative. Freud showed that the injection of adequate amounts of muscarinic drugs into rats caused the secretion of opaque reddish-brown tears. Tashiro, Badger, and Younker (1940) showed that the pigment was a chloroform soluble substance giving a strong fluorescence in ultra-violet light, and this material was identified by Figge (1945) spectrographically as consisting mainly of proto-porphyrin with a small amount of coproporphyrin I. These porphyrins are evidently the secretion of the Harderian gland, which was shown by Derrien and Turchini (1924) to have glandular cavities lined by cells whose vacuoles contained a pigment showing the same characters as the red tear pigment. Figge found that, of a large number of animals examined, only rats, Syrian hamsters, and some strains of mice (e.g., C_3H strain but not JK strain) produced this secretion. Tashiro, Smith, Badger, and Kezur (1940) found that eserine reduced the amount of acetylcholine that was required to produce a response very greatly, and Mendel and Hawkins (1943) showed that injection of cholinesterase into rats abolished the response to normal doses of acetylcholine.

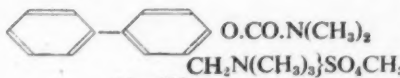
This paper describes how this response may be used as a basis for an accurate and quantitative assay of anticholinesterase drugs.

MATERIALS

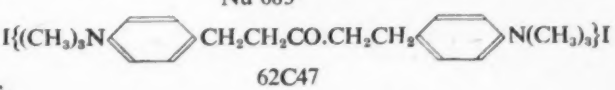
Male albino Wistar rats weighing 100–200 g. were found to give the most satisfactory and consistent results. They were divided into groups of six rats whose weights fell within the range of ±10–15 g. from the mean. The standard acetylcholine solution was prepared by dissolving 100 mg. acetylcholine chloride and 100 mg. KH_2PO_4, 12 H_2O in 100 ml. of a 0.8 g./100 ml. solution of NaCl. This solution has a *p*H of 4.1–4.3 and keeps without loss of activity in the refrigerator for a few weeks.

Tetraethylpyrophosphate (TEPP) was the pure thrice-distilled ester (sp. gr. 1.25) which on hydrolysis with NaOH yielded 98–99 per cent of the theoretical acid. "Hexaethyltetraphosphate" (HETP) was a mixture of alkyl polyphosphates prepared by heating 1 mol of P_2O_5 with 2 mols of triethylphosphate at 140° C. for one hour. Both these substances are hydrolysed in aqueous solution and hence fresh aqueous solutions were prepared immediately before use. Di*iso*propyl fluorophosphonate (DFP) was the pure ester kept as a 2 per cent (w/v) stock solution in dry propylene glycol. Fresh aqueous solutions were prepared immediately before use.

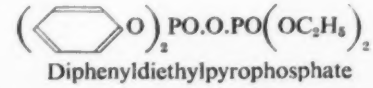
The other anticholinesterase drugs studied were: eserine sulphate, neostigmine methylsulphate, the dimethylcarbamic ester of 5-phenyl-2-hydroxybenzyl-trimethylammonium bromide (Nu 683), bis-(4-dimethyl-aminophenylethyl)ketone dimethiodide (62C47 Glock and Mogey, 1948), quinidine sulphate, and diphenyl-diethylpyrophosphate.



Nu 683



62C47



Diphenyldiethylpyrophosphate

Methods

Injections of acetylcholine were made subcutaneously under the loose skin of the rat's flank. When 300–500 μg. acetylcholine were injected a secretion of reddish-brown opaque tears commenced in 30–60 sec.; this could be tested by inserting a spill of filter paper into the conjunctival sac when the pigment was readily visible on the filter paper; when the secretion was profuse, it welled out of the conjunctival sac and formed an opaque red pool covering the eyeball and smearing the fur covering the eyelids; it could also often be seen at the nostrils. The secretion continued for about 4–6 min. as a rule, but after administration of anticholinesterase drugs it tended to be more prolonged. If the amount of acetylcholine injected was decreased the secretion appeared somewhat later (1½–2 min.), and it was usually preceded by colourless tears (lacrimal gland secretion only) and was less intensely coloured; finally, if the amount of acetylcholine was still further reduced either no secretion or colourless tears only were formed. In different groups of rats tested over the past fifteen months the threshold amount required to produce red tears has been in the range of 80–250 μg. acetylcholine; for a given group of rats the range of sensitivity has usually been narrow, the mean threshold dose varying by only ±25–30 μg. Occasionally rats reacted well outside this range, and they were consequently rejected. The absence of one or both Harderian glands is not a very rare occurrence in rats, and consequently it is essential that both conjunctival sacs be tested. For practical purposes the threshold figure adopted has been that dose at which half the animals gave a positive response rather than the mean of the individual thresholds for each animal, and in what follows the term threshold will be used with this connotation. In a given group of animals the threshold has shown no change over several weeks, and it may be tested as frequently as every 30 min. without exhaustion of the gland's secretory capacity. The presence of secretion has always been tested for 1½ min. after injection and if negative again at 3 min. If an animal shows no secretion at 3 min. it is most unlikely that any secretion will appear. The anticholinesterase drugs tested were administered by intraperitoneal injection.

The inhibition by the drugs of true cholinesterase, from haemolysed human red blood cells, and of pseudo-cholinesterase, from human oxalate plasma, was tested with acetyl-β-methylcholine bromide and benzoylcholine chloride respectively as substrates, as will be described elsewhere (Burgen, 1949).

An actual assay is carried out as follows: the threshold of six animals, in the absence of anticholinesterase drugs, is first determined; after being left in their cage for 30 min. the animals are injected intraperitoneally with the cholinesterase inhibitor. If the time of maximum activity of the drug is known the threshold of the animals need only be tested at this time. If, however, the time course of the drug is unknown two or three groups of six rats are used; these are tested at suitable intervals, such that the same group of rats is never tested more frequently than once in thirty minutes. In this way sufficient values can be obtained to give the complete time-effect curve and thereafter further doses need only be tested at the time of maximum activity. It has been our custom to test the response of every new batch of rats to 0.25 mg. eserine sulphate per kg. both as a test of the constant sensitivity of the rats used and so as to have available figures for both absolute potencies and potencies relative to eserine sulphate.

Results

Effects of eserine

After the injection of 0.2–0.3 mg. eserine sulphate per kg. the threshold to acetylcholine began to fall after 2–3 min., reached a minimum after 18–25 min. (Fig. 1), and then began to return towards

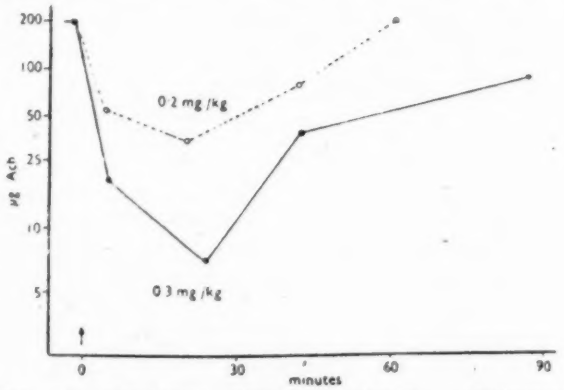


FIG. 1.—Abscissae: time in minutes. Ordinates: amount of acetylcholine needed to produce red tear secretion. At the arrow eserine sulphate was injected intraperitoneally. o----o 0.2 mg./kg; ● —— ● 0.3 mg./kg.

the initial level. It will be seen that after the administration of 0.2 mg. eserine sulphate per kg. the threshold fell from 200 μg. to 35 μg. acetylcholine, whereas with 0.3 mg. eserine sulphate per kg. the threshold was lowered to 7 μg. acetylcholine. Fig. 2 shows the dose-response curve covering the effective range of eserine dosage which is linear when the ordinate is plotted as the

$$\log_{10}\left(\frac{\text{Initial acetylcholine threshold}}{\text{Minimum threshold after the drug}}\right)=\log_{10}\left(\frac{To}{Tm}\right)$$

The response curve is steep, so that a change of 10 per cent in the dose of eserine corresponds to a change in acetylcholine threshold of c. 150 per cent. Repeated tests on the same group of rats have shown that the standard deviation of the mean of a potency determination on eserine is c. ± 3 per cent. It was found that with doses of an anticholinesterase drug which reduced the threshold below 5 μg. acetylcholine the end point became difficult to determine as the tears were very watery and poorly pigmented. Evidently in this range of dosage the anticholinesterase drugs

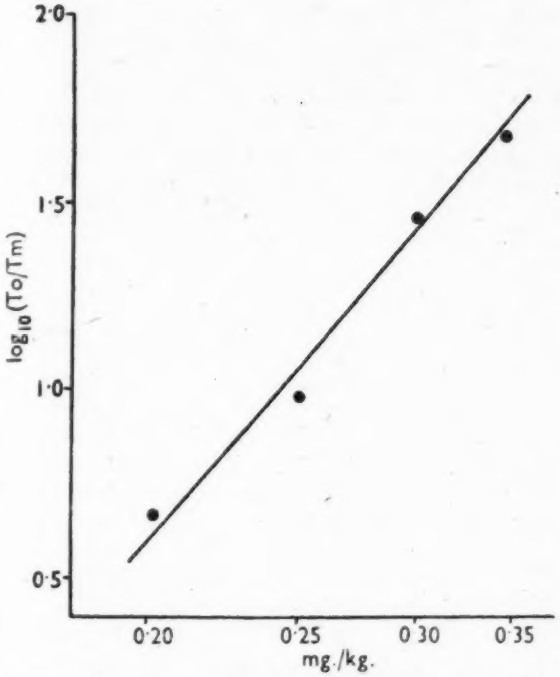


FIG. 2.—Abscissae: dose of eserine sulphate mg./kg. Ordinates: $\log_{10}\frac{To}{Tm}$, where To = initial red tear threshold to acetylcholine and Tm = minimum threshold reached after administration of eserine.

produce a relatively greater sensitization of the lacrimal gland than of the Harderian gland.

Eventually if the amount of anticholinesterase drug was increased sufficiently a spontaneous secretion occurred without the injection of acetylcholine, presumably owing to the acetylcholine released at the cholinergic nerve endings in the gland.

Comparisons with other drugs

Other anticholinesterase drugs have given dose-response curves with slopes similar to that of eserine, but displaced along the abscissae according to the relative potencies. Fig. 3 shows the curves obtained for four other drugs compared with that of eserine. A line has been drawn at the dose level corresponding to a tenfold potentiation of acetylcholine—i.e., the point at which $\log_{10}\left(\frac{To}{Tm}\right)=1.0$. The negative logarithm of the molar concentration of drug at which this potentiation was attained is called the pT10 and has been found to be a convenient measure of relative potencies.

The Table gives potency figures determined in this way for a number of known anticholinesterase drugs; data for DFP are not included because it was not found possible to produce a tenfold potentiation of

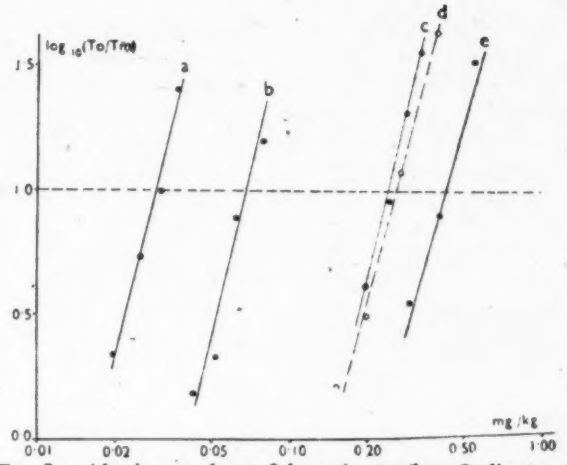


FIG. 3.—Abscissae: dose of drugs in mg./kg. Ordinates: $\log_{10}\frac{To}{Tm}$. a = neostigmine methylsulphate, b = "62C47," c = eserine sulphate, d = TEPP, and e = "Nu 683." The horizontal line is the tenfold potentiation level.

the response to acetylcholine by a single intraperitoneal dose of this drug; a twofold potentiation of the response was obtained with 0.6 mg./kg., but 0.8 mg./kg. was lethal to all the animals of the group studied. This illustrates a point in the series studied; with "62C47" the LD50 was about a hundred times as large as the tenfold potentiating dose; with eserine, neostigmine, and Nu 683 between ten and twentyfold; but with TEPP and HETP only about twice as great; whilst with DFP the tenfold potentiating dose could not be attained. With the short-acting drugs tests of potency could be performed on successive days, but with TEPP and HETP the animals responded normally to eserine only after four to seven days; in order to eliminate any error from this cause they were not used again for assay purposes in under fourteen days.

TABLE

Name and molecular weight	Potency (eserine = 100)		pT10
	By weight	Molar	
Eserine sulphate (324) ..	100	100	6.11
Neostigmine methyl sulphate (322)	860	860	7.05
Nu 683 (393)	59	71	5.96
TEPP (290)	100	89	6.10
HETP (—)	24	—	—
62C47 (610)	360	670	6.95
Quinidine sulphate (391)	<1	<1	<4.00
Diphenyldiethylpyrophosphate (386)	<1	<1	<4.00

Duration of effects

As previously noted the effect of eserine wore off in two to three hours, depending on the dose administered; neostigmine and "Nu 683" had similar

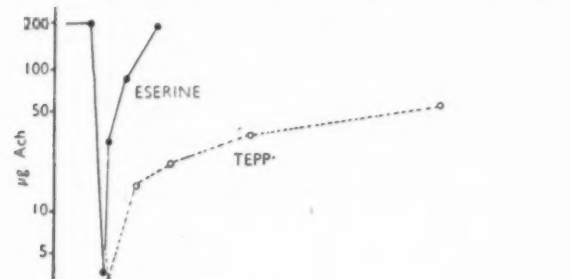


FIG. 4.—Abscissae: time in hours. Ordinates: threshold doses of acetylcholine. Anticholinesterase drug injected at zero time. ●——● eserine sulphate 0.32 mg./kg ; o----o TEPP 0.3 mg./kg.

durations, and "62C47" a briefer effect lasting only one to two hours TEPP and HETP, however, reached their peak action only after forty to fifty minutes, and their effect declined more slowly (Fig. 4), so that after twelve hours a marked potentiation was still present and even after thirty-six to forty-eight hours a small degree of potentiation was still present. The relatively slow development of the action of TEPP appears to be the result of a slow reaction between TEPP and cholinesterase and not to delayed absorption (Jansen, Nutting, and Balls, 1948; Burgen, 1949). Such effects as were obtainable with DFP reached a maximum in twenty-four to forty-eight hours and were still detectable seven days later.

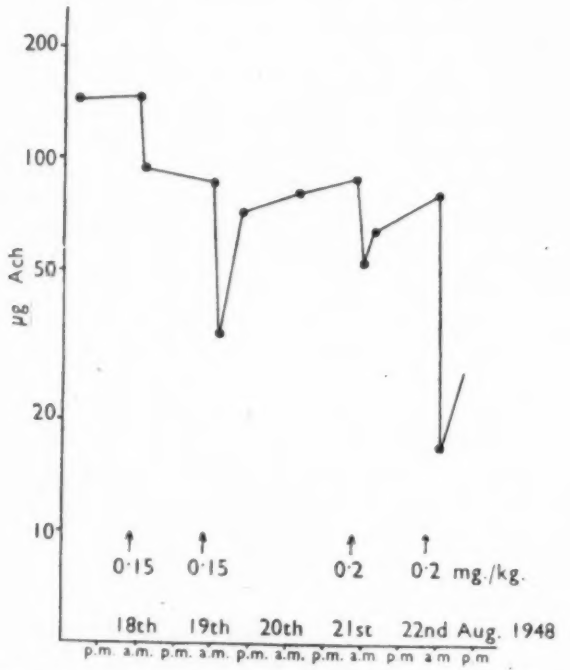


FIG. 5.—Abscissae: date. Ordinates: acetylcholine threshold. At arrows 0.15 mg./kg. and 0.2 mg./kg. TEPP injected intraperitoneally.

Cumulative effect of TEPP

Attempts to demonstrate a cumulative effect of TEPP by this assay have not been easy to interpret, but Fig. 5 shows some data obtained at levels of 0.15 mg./kg. and 0.20 mg./kg.; it will be seen that at both levels when the dose of TEPP was repeated after twenty-four hours a larger effect was produced than after the first dose.

Correlation with in vitro *inhibition of cholinesterase*

Some of the reasons why any close agreement between *in vitro* and *in vivo* potencies of anticholinesterase drugs of diverse modes of action is unlikely will be considered elsewhere (Burgen, 1949). Fig. 6, however, does show a general correspondence

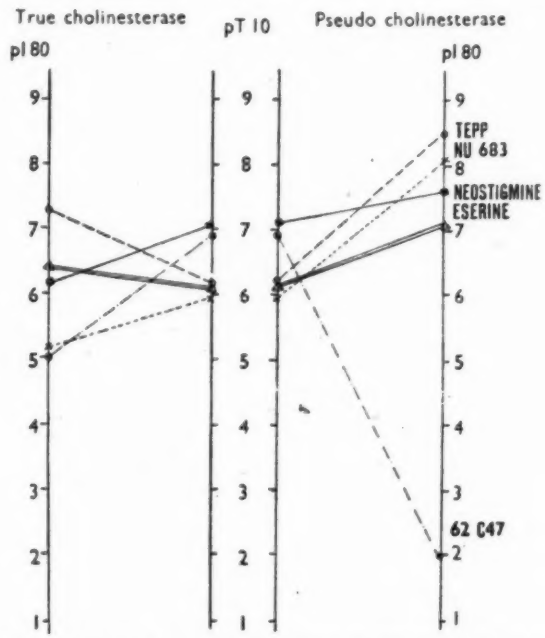


FIG. 6.—Figures in centre column pT10 determined by chromodacryorrhoea (see Table). Left-hand column pI80 for true cholinesterase. Right-hand column pI80 for pseudo-cholinesterase. △==△ eserine sulphate; ●——● neostigmine methylsulphate; ●——● "62C47"; ×·····× "Nu 683"; o----o TEPP.

between the potencies found by the chromodacryorrhoea method and those found *in vitro*. In this figure the pT10, determined as described earlier, is compared with the negative logarithm of the molar concentration of inhibitor required to inhibit true and pseudo cholinesterases *in vitro* to the extent of 80 per cent (pI80). An inhibitory level of 80 per cent was selected for comparison because there was evidence (Gunter and Mendel, 1945; Hawkins and Gunter, 1946; Koelle and Gilman, 1946; Hawkins and Mendel, 1947) to suggest that this is approximately the level at which cholinesterase inhibition becomes physiologically effective. These figures should therefore be numerically comparable; theoretically the lines joining the points should be nearly horizontal and this is in fact approximately true with the marked exception of the pI80 for pseudocholinesterase inhibition by "62C47."

Effect of atropine on chromodacryorrhoea response

Atropine in as small a dose as 20 μg./kg. raises the threshold to acetylcholine. In consequence the chromodacryorrhoea response must be regarded as a very sensitive test for atropine.

Discussion

It has been shown that the chromodacryorrhoea response is a sensitive quantitative indicator of the activity of anticholinesterase substances *in vivo*. It is clear that the protection of acetylcholine against hydrolytic destruction tested in this way can be divided into protection (1) during transport in the blood stream to the Harderian gland and (2) in the gland substance itself. There can be little doubt that destruction in the blood is the more important of the two factors, and consequently it becomes of importance to inquire as to the nature of rat serum esterase. In male rats most of the serum cholinesterase is true cholinesterase (Hawkins and Mendel, 1947), although in female rats a considerable amount of pseudocholinesterase occurs (Brauer and Root, 1947), so that effects in our tests would be expected to correlate better with inhibition of the true cholinesterase than of the pseudo-cholinesterase. This has been true, and it is illustrated not only by the weak action of DFP and by the lack of action of quinidine (see Table), both selective pseudo-cholinesterase inhibitors, but also by the powerful action of "62C47," a selective inhibitor of the true cholinesterase. It would seem therefore that the chromodacryorrhoea response in male rats is a test mainly of the ability of the drugs to inhibit true cholinesterase. Another important aspect of the test is that, with drugs that show some other marked pharmacological property besides anticholinesterase activity, it allows an assessment of the contribution of this activity in the whole animal.

Summary

The potency and the duration of action of anticholinesterase drugs have been measured in the unanaesthetized rat by the chromodacryorrhoea response. An approximate correspondence was obtained with their potencies as inhibitors of true cholinesterase *in vitro*.

I should like to express my thanks to Dr. C. A. Keele for kindly criticism and to Mr. B. Topley, Dr. G. A. Mogey, and Dr. F. Bergel for gifts of some of the inhibitors.

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THE SPECIFICITY OF THE TRIMETHYLAMMONIUM GROUP IN ACETYLCHOLINE

BY

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(Received February 21, 1949)

It has long been known that replacement of the three N-methyl groups of acetylcholine, or of other choline esters and ethers, by ethyl or larger alkyl radicals leads to virtual disappearance of stimulant actions on structures with parasympathetic innervation and at ganglion cells; such triethylammonium compounds retain only a paralysing action on ganglion cells (Hunt and Renshaw, 1929, 1933). The similar contrast between tetramethylammonium and tetraethylammonium salts is equally striking and well known (Burn and Dale, 1914), and studies of a more extensive range of " onium salts " suggest that only those containing at least three methyl groups attached to the central atom (which may be N, P, As, or S) retain muscarine-like and stimulating nicotine-like actions (Hunt and Renshaw, 1925; Ing and Wright, 1933). More recently Fourneau, Bovet, Bovet, and Montezin (1944) found that the intense muscarine-like properties of the acetal of 2 : 3-dihydroxypropyltrimethylammonium iodide were entirely abolished when the trimethylammonium group was replaced by triethylammonium. At the same time observations have been recorded which suggest that the integrity of the trimethylammonium group may not be indispensable to the possession of some stimulant actions on parasympathetic endings and on ganglion cells. Thus, Hunt and Taveau (1911) observed that β-acetoxyethyl*iso*amyldimethylammonium chloride (I; R = *iso*amyl) caused a fall (but sometimes a rise) of blood pressure in the cat, which was annulled by atropine; Hunt and Renshaw (1929) made similar observations with β-acetoxyethylbenzyldimethylammonium chloride (I; R = benzyl); both compounds were only feebly active. Hunt and Taveau also described a muscarine-like effect on the circulation of the compound (II), in which one methyl group of acetylcholine is replaced by acetoxyethyl, and some years later Hunt and Renshaw (1929) described a similar effect produced by tetra-β-hydroxyethylammonium chloride (III).

$$CH_3CO_2CH_2CH_2NMe_2R\}Cl \quad (I)$$

$$(CH_3CO_2CH_2CH_2)_2NMe_2\}Cl \quad (II)$$

$$(HO.CH_2CH_2)_4N\}Cl \quad (III)$$

In order to throw more light upon the specificity of the trimethylammonium group in choline derivatives we have studied the effect of successive replacement of the N-methyl groups of acetylcholine by (i) ethyl groups and (ii) acetoxyethyl groups. The names and formulae of the compounds investigated, and the symbols which will be used in referring to them, are given in Table I.

The effect of replacing the methyl groups of acetylcholine successively by hydrogen was studied

TABLE I

Name	$CH_3CO_2CH_2CH_2$ N^+ R	R′	R″	Symbol
Acetylcholine bromide ..	CH_3	CH_3	CH_3	Ach
Acetoxyethyldimethylethylammonium iodide	CH_3	CH_3	C_2H_5	MonoE
Acetoxyethylmethyldiethylammonium iodide	CH_3	C_2H_5	C_2H_5	DiE
Acetoxyethyltriethylammonium bromide ..	C_2H_5	C_2H_5	C_2H_5	TriE
Bis-acetoxyethyldimethylammonium iodide	CH_3	CH_3	$CH_3CO_2C_2H_4$	BisA
Tri-(acetoxyethyl)methylammonium iodide	CH_3	$CH_3CO_2C_2H_4$	$CH_3CO_2C_2H_4$	TrisA

TABLE II

Preparation and effect	Approx. equipotent molar ratios					
	Ach	MonoE	DiE	TriE	BisA	TrisA
Cat; fall of B.P.	1	3	400	>2,000	150	>20,000
Rise of B.P. after atropine	1	>5	No rise	No rise	No rise	No rise
Guinea-pig ileum; contraction	1	2.5	700	1,700	100	>20,000
Frog's heart; slowing and reduction of beat	1	2	1,500	Reduces Ach effect	75	12,500
Rabbit auricles; slowing and reduction of beat	1	1.6	600	Reduces Ach effect	50	25,000
Frog's rectus abdominis; contracture	1	5	300	5,000	164	No contracture; reduces Ach effect

by Stehle, Melville, and Oldham (1936), and their results will be considered later.

Materials

The following compounds were prepared:

(1) *Acetoxyethyldimethylethylammonium iodide*, by addition of ethyl iodide to dimethylaminoethyl acetate; crystallized from hot acetone by addition of ethyl acetate; m.p. 81–82°. (Found: C, 33.8; H, 6.2. $C_8H_{18}O_2NI$ requires C, 33.5; H, 6.3 per cent.)

(2) *Acetoxyethylmethyldiethylammonium iodide*, by addition of methyl iodide to diethylaminoethyl acetate; crystallized from methylethyl ketone; m.p. 68°. (Found: C, 35.7; H, 6.7. $C_9H_{20}O_2NI$ requires C, 35.9; H, 6.6 per cent.)

(3) *Acetoxyethyltriethylammonium bromide*, by addition of ethyl bromide to diethylaminoethanol and acetylation of the product; crystallized from acetone; m.p. 131–132°. (Found: C, 44.9; H, 8.1. $C_{10}H_{22}O_2NBr$ requires C, 44.8; H, 8.2 per cent.)

(4) *Bis-acetoxyethyldimethylammonium iodide*. Diethanolamine was methylated with formalin and formic acid and the product acetylated; addition of methyl iodide to the crude ester gave a product which crystallized from acetone, m.p. 113–114°. (Found: C, 34.8; H, 5.8. $C_{10}H_{20}O_4NI$ requires C, 34.7; H, 5.7 per cent.)

(5) *Tri-(acetoxyethyl)methylammonium iodide*, by acetylation of triethanolamine and addition of methyl iodide to the crude ester; crystallized from acetone, m.p. 110–111°. (Found: C, 37.8; H, 5.8. $C_{13}H_{24}O_6NI$ requires C, 37.4; H, 5.8 per cent.)

All these compounds are hygroscopic. Hunt and Taveau (1911) prepared the chloroplatinates of acetoxyethyltriethylammonium and bis-acetoxyethyldimethylammonium.

Methods

Each substance was compared with acetylcholine on the following preparations:

(1) Blood pressure of the cat under chloralose.
(2) Isolated ileum of the guinea-pig.
(3) Perfused heart of the frog.
(4) Isolated auricles of the rabbit.
(5) Rectus abdominis of the frog.

TriE and TrisA were also tested on the perfused superior cervical ganglion of the cat. TriE was tested for mydriatic activity in mice by Pulewka's method (1932; cf. Ing, Dawes, and Wajda, 1945). TrisA was tested on the denervated gastrocnemius of the cat. In addition the hydrolysis of each substance by the cholinesterases of horse serum, dog's caudate nucleus, and cobra venom was studied manometrically.

Results

The results obtained with the first five preparations mentioned above are summarized in Table II; they are expressed as equipotent molar ratios and the figures are therefore inversely proportional to molar activities. The effects of the substances were qualitatively similar to those of acetylcholine unless otherwise stated.

The equipotent molar ratios were obtained either by matching doses or by plotting the magnitude of an effect against the logarithm of the dose; they are only approximate because some of the preparations were not sensitive enough to distinguish between doses differing by less than 50 per cent. Moreover, the responses to increasing doses of acetylcholine were usually more steeply graded than those to increasing doses of the other compounds, and consequently an arbitrary response had to be chosen for the estimation of equipotent doses; thus with the rectus abdominis a contracture 50 per cent of the maximal was chosen. With the rabbit's auricles linear and parallel relations between percentage inhibition and log. dose were obtained for Ach, MonoE, and DiE.

The actions of MonoE, DiE, and BisA.—The effects of these three substances resembled those of acetylcholine closely, except that a nicotine-like rise of blood pressure after atropine was not obtained with DiE (4 mg.) or BisA (2 mg.). The replacement of one methyl group of acetylcholine by an ethyl group produces a relatively small decrease in activity, MonoE being between a half and a third as active

as Ach in its muscarine-like effects and about a fifth as active in its nicotine-like effects; but the replacement of two methyl groups by ethyl produces a decline in activity of an entirely different order, DiE having on the average about one five-hundredth of the activity of acetylcholine.

The introduction of a second acetoxyethyl group in place of methyl into acetylcholine is a more radical chemical change than the replacement of one methyl by ethyl and it produces a more pronounced decrease in activity, BisA being about one-hundredth as active as acetylcholine, but still much more active than DiE.

The action of TriE.—The replacement of all three methyl groups of acetylcholine by ethyl reduces the activity on all five preparations by a factor of at least a thousand; moreover it alters the kind of activity on the heart and circulation. In cats under chloralose large doses of TriE (20 mg.) produced a fall of blood pressure which developed more slowly and was more prolonged than that produced by Ach and could be obtained after atropine. Similar doses also reduced the pressor effect of small doses of nicotine. It seems probable that the fall of blood pressure is due to a paralysing action on sympathetic ganglia. Hunt and Renshaw (1929, 1933) also concluded that TriE had a paralysing nicotine-like action; they used cats in which the C.N.S. had been destroyed and, probably for this reason, they observed no fall in blood pressure, but after atropine TriE diminished or annulled the pressor effect of tetramethylammonium. In order to demonstrate this paralysing nicotine-like action the effect of TriE on the perfused superior cervical ganglion of the anaesthetized cat was investigated. As Fig. 1 shows TriE in doses of 1–10 mg. abolished or reduced the contractions of the nictitating membrane in response to preganglionic stimulation, but the effect was short-lived. It is interesting that although TriE had no stimulant effect on sympathetic ganglia it did cause contracture of the frog's rectus abdominis; it did not antagonize Ach in this muscle, nor did it show any curariform action on the rat's phrenic nerve-diaphragm in concentrations so high as 30 mg./100 ml. (one experiment).

Hunt and Renshaw observed no reduction in heart rate in their experiments with TriE; in our experiments also the heart rate in anaesthetized cats was unaltered by TriE. TriE had no effect on the rate of the perfused frog's heart, but it did prevent or reduce the effect of subsequent doses of Ach; when 0.1 ml. $10^{-6}M$ Ach was given at one-minute intervals, 0.1 ml. $10^{-1}M$ TriE prevented the effect of Ach for two minutes and the full effect was only restored five minutes after the administration of TriE. Smaller doses of TriE had a similar effect but o shorter duration (Fig. 2). Somewhat similar effects were also observed with the isolated rabbit auricles, although large doses were required; e.g., 8 mg. TriE nearly but not quite abolished the effect of 11 μg. Ach. It is doubtful whether these blocking

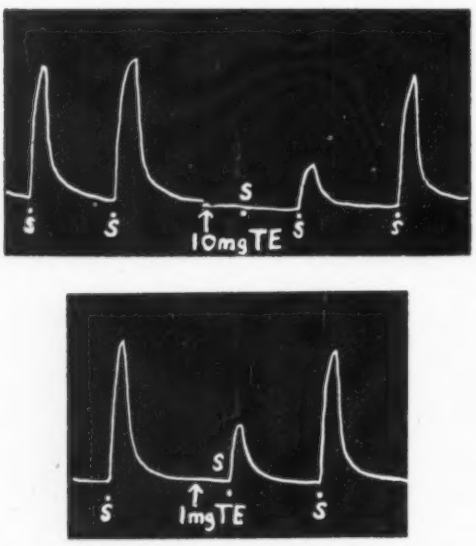

FIG. 1.—Perfused superior cervical ganglion of cat under chloralose. Record of contractions of nictitating membrane in response to stimulation of preganglionic fibres for 4 sec. every 3 min. at S. TriE (given at TE) reduced the response to stimulation.

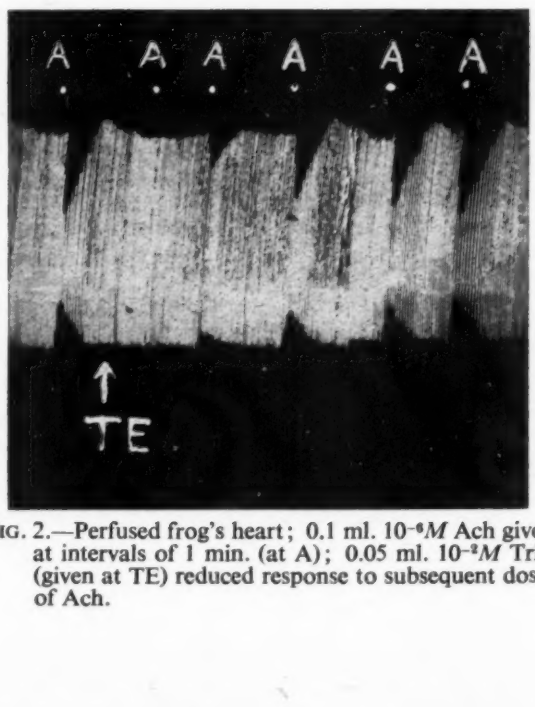

FIG. 2.—Perfused frog's heart; 0.1 ml. $10^{-6}M$ Ach given at intervals of 1 min. (at A); 0.05 ml. $10^{-2}M$ TriE (given at TE) reduced response to subsequent doses of Ach.

effects are truly atropine-like, because TriE did not reduce the effects of Ach on the cat's blood pressure or the guinea-pig's ileum; on the latter TriE had a weak stimulant action. An attempt was made to discover whether TriE had any mydriatic activity, but the results were inconclusive. Groups of five mice were injected intraperitoneally with varying doses of TriE and the pupil diameters measured at intervals. Some dilatation was observed but only after toxic doses; thus a fivefold mean dilatation was obtained with 2 mg. TriE per mouse, but three out of five mice died; 1 mg. TriE produced a threefold dilatation but killed one out of five mice.

The action of TrisA.—The introduction of two acetoxyethyl groups in place of methyl into acetylcholine reduces activity on the five preparations by a factor of at least ten thousand. Nevertheless the effects of TrisA on the cat's blood pressure, the guinea-pig's ileum, and the frog's heart are truly

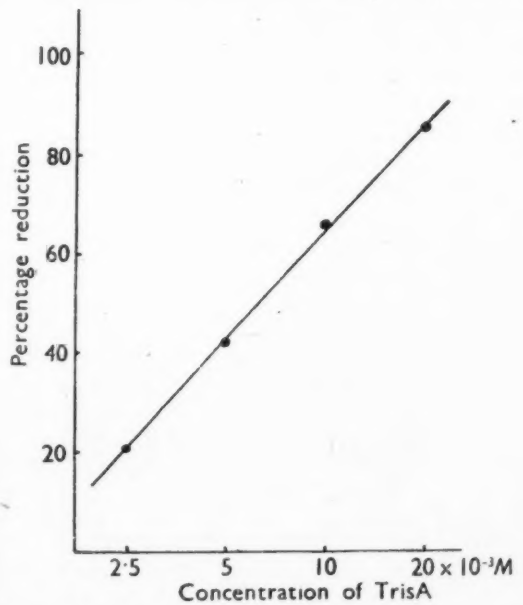

FIG. 3.—Antagonism between TrisA and Ach on rectus abdominis (frog). Ordinates: percentage reduction in contractures produced by 0.75 × 10^{-6}*M* Ach. Abscissae: molar concentration of TrisA: logarithmic scale.

muscarine-like since they are reduced or abolished by atropine. TrisA did not appear to have any stimulant nicotine-like actions, either on the blood pressure or on striated muscle; it produced no contracture of the frog's rectus abdominis, but it antagonized the effect of Ach on this muscle, and this antagonism was linearly proportional to the logarithm of the concentration of TrisA (Fig. 3).

Similarly TrisA reduced the effect of Ach on the cat's denervated gastrocnemius. Fig. 4 is a record of the contractions of a cat's gastrocnemius, which had been denervated eight days previously; A_1, A_2, and A_3 were contractions in response to the injection of 10 μg. Ach into the iliac artery; at TS 25 mg.

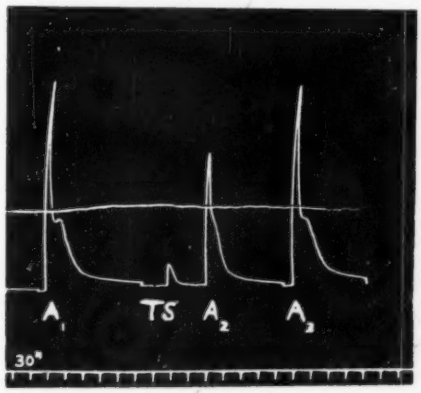

FIG. 4.—Cat's denervated gastrocnemius. Record of contractions in response to 10 μg. Ach injected into iliac artery at A_1, A_2, and A_3; 25 mg. TrisA, injected at TS, reduced response to next injection of Ach.

TrisA were injected; one minute elapsed between TS and A_2, eight minutes between TS and A_3. It will be seen that TrisA produced a small contraction itself but reduced the response to the next injection of Ach by about 40 per cent.

A similar kind of effect was observed in the cat's superior cervical ganglion, perfused with Locke's solution (Fig. 5): injection of 8 mg. TrisA produced no contraction of the nictitating membrane, but diminished its response to stimulation of the

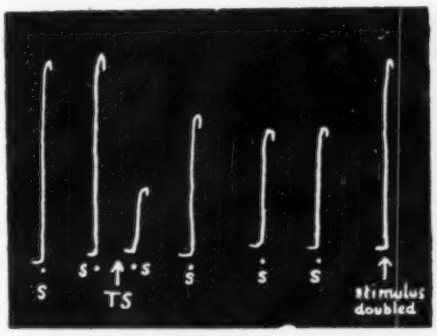

FIG. 5.—Perfused superior cervical ganglion of cat under chloralose. Record of contractions of nictitating membrane in response to preganglionic stimulation at S. At TS 20 mg. TrisA injected 20 sec. before next stimulus.

preganglionic fibres 20 seconds later; the intensity of the stimulus had to be doubled in order to obtain a contraction as large as before the injection of TrisA.

Enzymic hydrolysis

All the substances were hydrolysed by horse serum and a preparation of dog's caudate nucleus; the rates of hydrolysis are given in Table III. It will be noticed that the mono-, di-, and tri-ethyl compounds were hydrolysed by dog's caudate nucleus at about the same rate as acetylcholine, but with horse serum the rate declined with successive substitution of methyl by ethyl groups. With BisA the volume of CO_2 evolved indicated that both ester groups were hydrolysed, but the hydrolysis of TrisA was too slow to be followed to completion.

Since a specimen of cobra venom was available the rates of hydrolysis of the compounds by this preparation were also measured, and the results are included in Table III.

TABLE III

COMPARISON OF RATES OF ENZYMIC HYDROLYSIS OF ESTERS

Substrate concentrations were $10^{-2}M$ for horse serum and $6 \times 10^{-2}M$ for dog's caudate nucleus and cobra venom

Substrate	μl. CO_2 evolved in 15 min. (no correction for retention)		
	0.2 ml. horse serum	4 mg. caudate nucleus	0.2 mg. cobra venom
Ach ..	61	49	87
MonoE	44	53	77
DiE ..	30	47	65
TriE ..	16	46	66
BisA ..	51*	52*	104*
TrisA ..	5	11	12

* Both ester groups hydrolysed

In order to make sure that the observed hydrolysis was due to the cholinesterases of horse serum and caudate nucleus the effect of specific inhibitors of these preparations on the hydrolysis was studied. The hydrolysis of each compound by horse serum was inhibited by the dimethylcarbamic ester of 2 - hydroxy - 5 - phenylbenzyltrimethylammonium bromide (Nu 683), which is a fairly specific inhibitor of the enzyme known as pseudo-cholinesterase (Hawkins and Gunter, 1946). The percentage inhibitions with $10^{-5}M$ inhibitor are given in Table IV; they suggest that the cholinesterase of horse serum is responsible for the hydrolysis of these esters. This conclusion was supported by observations with a purified preparation of horse serum cholinesterase, which hydrolysed all five esters, as well as acetylcholine, at approximately the same relative rates as the unpurified horse serum. We are indebted to Dr. J. W. Legge for this enzyme preparation.

TABLE IV

THE EFFECT OF SPECIFIC INHIBITORS OF CHOLINESTERASES

Substrate	Percentage inhibition of	
	horse serum with $10^{-5}M$ Nu 683	caudate nucleus with $3 \times 10^{-6}M$ eserine
Ach ..	71	90
MonoE ..	74	93
DiE ..	68	87
TriE ..	58	95
BisA ..	69	82
TrisA ..	57	80

Table IV also includes data on the percentage inhibition of the hydrolysis of these esters by dog's caudate nucleus in the presence of 3×10^{-6} eserine. These observations indicate that the esters were also substrates of the enzyme known as true cholinesterase.

In accordance with these manometric experiments it was observed that the effects of the five esters on the cat's blood pressure were all increased and prolonged by the previous administration of a large dose (0.5 mg.) of eserine.

Discussion

The main conclusions to be drawn from the results described above are: that the presence of three N-methyl groups is not indispensable to the typical pharmacological properties of acetylcholine; that replacement of one methyl group by ethyl produces a relatively small decrease in activity; but that replacement of two methyl groups by ethyl leads to a profound decline in activity. Similarly, although the replacement of one methyl group by acetoxyethyl reduces activity more than replacement by ethyl, the substitution of a second methyl group by acetoxyethyl has proportionately a very much greater effect. These conclusions suggest that two N-methyl groups play an important part in the structural specificity of molecules of this type.

The specificity of the acetoxy group is well known, and if it be assumed that at the site of its action acetylcholine is attached (e.g., by van der Waals forces) to some component molecule of the excitable structure not only by this group but also by two methyl groups, and the tetrahedral arrangement of the 4-covalent nitrogen atom would allow this, then replacement of one methyl group by ethyl will

reduce the probability of the correct orientation for attachment but will not make it impossible, as the replacement of two methyl groups by ethyl would do. The probability of attachment of an NMe_2R-group will not depend solely upon the geometry of the structure—if it did, it would be one-third that of the NMe_3- group—but it is impossible to predict to what extent the radical R, which may well have its own affinity for the excitable structure, will influence the ease of attachment. We have already noted that replacement of one methyl in acetylcholine by acetoxyethyl diminishes activity much more than replacement by ethyl; replacement by benzyl (Hunt and Renshaw, 1929) appears to reduce the activity of the molecule (on the cat's blood pressure) to about one-thousandth that of acetylcholine, and Hunt and Taveau (1911) described acetoxyethyldimethyl*iso*amylammonium chloride as comparable in activity with choline. The effect of replacement by hydrogen is interesting; Stehle, Melville, and Oldham (1936) studied the effect of successive replacement of the methyl groups of acetylcholine by hydrogen, and their results have been converted in Table V into the form adopted by us for recording our own results in Table II. The progressive decline in activity as methyl groups are successively replaced by hydrogen is not unexpected, but it will be noticed that the effect of replacing one methyl by hydrogen is at least ten times greater than that of replacing one methyl by

TABLE V

Data from Stehle, Melville, and Oldham (1936)

Substance	Approx. equipotent molar ratios		
	Cat's blood pressure	Rabbit intestine	Frog's heart
$AcO.C_2H_4NMe_3\}Cl$ ..	1	1	1
$AcO.C_2H_4NMe_2H\}Cl$ ..	50	>40	>50
$AcO.C_2H_4NMeH_2\}Cl$ ..	>500	>1,000	>500
$AcO.C_2H_4NH_3\}Cl$ ..	>2,000	20,000	40,000

ethyl; the former change, however, alters the character of the cation, which ceases to be a stable structure.

The form of attachment envisaged in our hypothesis—viz., by means of short-range atomic forces—implies a high degree of fit between the drug molecule and the site of action, and structural changes which, for example, alter the mean distance between the methyl groups might be expected to decrease the probability of attachment and hence the activity of the drug. This effect is illustrated by the results of Welch and Roepke (1935) with the phosphorus and arsenic analogues of acetylcholine. In Table VI we have converted their figures for molar potency into the form adopted in Table II. It will be seen that, for muscarine-like effects, acetylphosphocholine is approximately one-twelfth, and acetylarsenocholine one-eightieth, as active as acetylcholine. The replacement of nitrogen by phosphorus or arsenic

TABLE VI

Data from Welch and Roepke (1935)

Substance	Approx. equipotent molar ratios			
	Cat's blood pressure	Rabbit's intestine	Frog's heart	Frog's rectus
$AcO.C_2H_4NMe_3\}Cl$	1	1	1	1
$AcO.C_2H_4PMe_3\}Cl$	13	12	12	6
$AcO.C_2H_4AsMe_3\}Cl$	66	90	83	37

will not alter the angle between the valencies, but owing to the larger size of the phosphorus and arsenic atoms it will increase the mean distance between the methyl groups. The best available values for the N–C, P–C, and As–C links in methyl compounds are 1.47, 1.87, and 1.98 Å respectively (Pauling, 1945). The tetrahedral angle between the valencies of 4-covalent N, P, or As is 109° 28′ and hence the distance between the carbon centres of two methyl groups will be $2y \sin 54° 44'$, where y is the length of the link between carbon and the central atom. The calculated distances are: for nitrogen, 2.40; for phosphorus, 3.05; and for arsenic, 3.23 Å. Thus in acetylphosphocholine the mean carbon-carbon distance between two methyl groups will be 27.1 per cent greater than in acetylcholine; in acetylarsenocholine it will be 34.6 per cent greater. It is interesting that acetylphosphocholine is less active than MonoE in spite of the presence of three methyl groups, and this may well be due to the greater separation of the methyl groups, an effect which is further intensified in acetylarsenocholine.

Another result of increasing the size of the cationic head by replacing the nitrogen atom by phosphorus or arsenic will be to diminish the Coulomb attraction between the cation and an anion in the excitable structure, but the potential function of this attraction is inversely proportional to the distance between the ions, and will consequently decrease much less rapidly with increase in the ionic radius than the van der Waals forces, which are inversely proportional to the sixth power of the distance between atoms.

Summary

1. The effect of successive replacement of the N-methyl groups of acetylcholine by (i) ethyl groups and (ii) acetoxyethyl groups has been studied on the cat's blood pressure, guinea-pig's ileum, the frog's heart, rabbit auricles, and the frog's rectus abdominis.

2. The replacement of one methyl group of acetylcholine by ethyl does not alter the pharmacological activities qualitatively and only reduces them to between a half and a fifth of those of acetylcholine. The replacement of two methyl groups by ethyl produces a drastic reduction in the activities, without altering their nature.

3. The replacement of all three methyl groups by ethyl alters the kind of pharmacological effect produced. Acetoxyethyltriethylammonium has paralysing nicotine-like properties on the cat's blood pressure and superior cervical ganglion; it also antagonizes acetylcholine in the frog's heart and the rabbit's auricles.

4. The replacement of one methyl group by acetoxyethyl does not alter the kinds of activity, but it reduces them more than replacement by ethyl. Replacement of two methyl groups by acetoxyethyl produces a very much greater decline in activities than replacement of one methyl group.

5. The mono-, di-, and tri-N-ethyl analogues of acetylcholine are hydrolysed by the cholinesterases of dog's caudate nucleus and cobra venom at about the same rates as acetylcholine. The rate of hydrolysis by the cholinesterase of horse serum declines with successive substitution of methyl by ethyl groups.

6. Both ester groups of bis-acetoxyethyldimethylammonium iodide are hydrolysed by the cholinesterases of horse serum, dog's caudate nucleus, and cobra venom. Tri-(acetoxyethyl)methylammonium iodide is hydrolysed much more slowly by all three enzyme preparations.

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SOME PHARMACOLOGICAL PROPERTIES COMMON TO ATROPINE, PETHIDINE, PROCAINE, AND QUINIDINE

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(Received March 14 1949)

In earlier papers from this laboratory attention has been drawn to the similarity in the pharmacological properties of substances which are used in medicine for quite different purposes. In examining compounds for a quinidine-like action, Dawes (1946) pointed out that quinine, quinidine, and procaine resemble one another in antagonizing acetylcholine in all forms of muscle. Dews and Graham (1946) showed that the antihistamine substance neoantergan was quinidine-like on heart muscle and had a local anaesthetic action like procaine. Elío (1948) examined the actions of atropine, procaine, and quinidine (and also other local anaesthetics) on the frog rectus, the rabbit intestine, and the rabbit vessels; he found that these substances antagonized the action of acetylcholine in all three preparations; he also observed a parallelism between the inhibiting action of these drugs on the frog rectus and their local anaesthetic action. This parallelism was extended to conessine and its derivatives by Stephenson (1948).

Another way in which the similarity of these substances was revealed was in their action on body temperature. Glaubach and Pick (1931) observed that procaine caused a fall of body temperature. I therefore studied (Dutta, 1948) the action of atropine, benadryl, pethidine, and quinidine, and found that they, like procaine, reduced body temperature in mice. Finally, Burn and Dutta (1948) have shown that these substances also resemble one another in being able to reverse the constrictor action of adrenaline on the vessels of the rabbit's ear.

The present paper describes the action of these substances on some other preparations; viz., on the perfused superior cervical ganglion of the cat, on the phrenic nerve-diaphragm preparation of the rat, and on the bronchioles of the guinea-pig. Thus they have been examined for curariform activity, both in the ganglion and at the motor endplate, and also for antihistamine activity.

Action on superior cervical ganglion

The superior cervical ganglion of the cat, anaesthetized with chloralose (80 mg./kg.), was perfused according to the method of Feldberg and Gaddum (1934) (originally introduced by Kibjakow, 1933). Locke's solution at 35° C. was introduced into the ganglion through the common carotid artery at a

TABLE I

ACTION ON SUPERIOR CERVICAL GANGLION

Exp. No.	Substance	Amount injected in μg.	Height of contraction of the nictitating membrane in mm.		Percentage inhibition
			Before addition of drug	After addition of drug	
5	Atropine sulphate	50	44	23	48
7	,,	50	52	0	100
8	,,	50	30	16	47
1	Pethidine hydrochloride	800	52	0	100
2	,,	200	46	39	15
2	,,	400	43	22	50
5	,,	600	42	31	26
8	,,	250	33	4	88
3	Quinidine hydrochloride	500	50	0	100
4	,,	500	38	22	42
5	,,	250	45	36	20
5	,,	500	39	8	80
3	Procaine hydrochloride	500	58	33	43
3	,,	750	58	0	100
5	,,	250	43	39	9
5	,,	400	43	32	26
7	,,	100	46	43	7
8	,,	250	41	18	56

perfusion pressure round about 110 mm. Hg, maintained with a small pump designed by Dr. E. J. Schuster. The preganglionic fibres were stimulated maximally at the rate of 16 per second for 10 seconds at 3-minute intervals. The contractions of the nictitating membrane were recorded with an isotonic lever. The substances were dissolved in Locke's solution, and not more than 0.2 ml. was injected at any time into the arterial cannula. Each of the compounds was tested on several preparations.

The results in different experiments are given in Table I, and an illustration of the action of pethidine, atropine, and quinidine is given in Fig. 1. Harvey

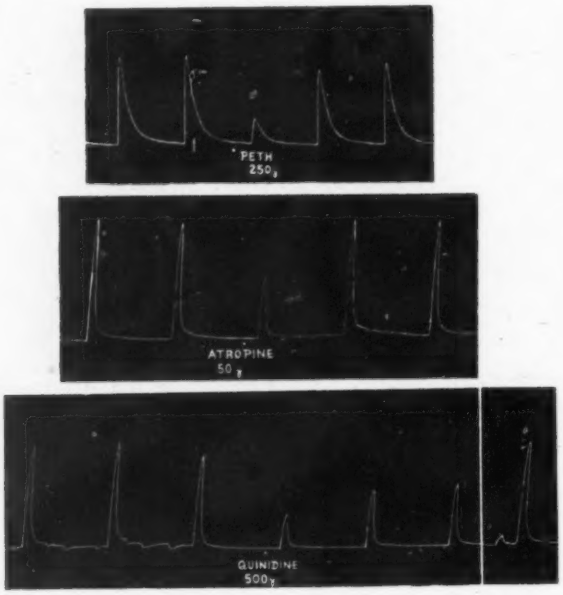

FIG. 1.—Superior cervical ganglion of the cat perfused with Locke's solution. Contractions of the nictitating membrane in response to preganglionic stimuli, 16 per sec. for 10 sec. every 3 minutes. Partial blocking of the ganglionic transmission by 250 μg. pethidine hydrochloride (upper tracing), 50 μg. atropine sulphate (middle tracing), and 500 μg. quinidine hydrochloride (lower tracing).

(1939a) has already shown that procaine diminishes the effect of preganglionic stimulation because of its action on the ganglion. The most powerful of the four substances was found to be atropine, which in a dose of 50 μg. abolished the effect of stimulation in one observation, and reduced the effect to half in two other observations in different experiments. Since *d*-tubocurarine chloride produces a similar effect in a dose of 5–10 μg., it follows that atropine has from 10–20 per cent of the inhibitory action of *d*-tubocurarine on the ganglion. From the results shown in Table I, it is difficult to give a figure for the other substances, though it is clear that they are weaker; pethidine and quinidine seem on the whole more active than procaine, but the effect of quinidine, illustrated in Fig. 1, is more prolonged.

Phrenic nerve-diaphragm preparation

The rat diaphragm method described by Bülbring (1946) was used; Tyrode solution containing double the amount of glucose was the bathing fluid and the 50-ml. bath was maintained at 37° C. and aerated with oxygen containing 5 per cent CO_2. The nerve was stimulated by a pair of platinum electrodes kept submerged in the solution. The muscle was excited directly with a pair of electrodes which consisted of insulated copper wires with silver tips. One electrode was placed near the centre of the base of the fan-shaped diaphragm, while the other was fixed at the apex of the muscle. Shocks were delivered from an electronic square wave stimulator at a desired strength, frequency, and duration.

The observations were most definite when pethidine was used; they are illustrated in Fig. 2 (*a*) to (*f*). When stimulation was applied through the nerve, the addition of pethidine to make a concentration of 10^{-4} resulted in an increase of muscle contractions by 67 per cent, as shown in (*a*). A similar effect was obtained when direct stimulation was used, as shown in (*b*). After the pethidine had been washed out again, 100 μg. *d*-tubocurarine chloride were added to the bath during the application of stimuli to the nerve and allowed to remain in contact until complete curarization was obtained (*c*). Direct stimulation was applied to the muscle; the addition of the same concentration of pethidine again increased the muscle contractions (*d*). This effect was obtained during full curarization, because when indirect stimulation was applied just before the bath was washed out there was at first no response. The earliest return of the response is shown at the right hand of the record (*d*).

Fig 2 (*e*) shows a similar increase caused by pethidine (4×10^{-5}), and Fig. 2 (*f*) shows the change from augmentation to depression when a much higher concentration (2.6×10^{-4}) was used. Both these effects (*e* and *f*) were obtained with nerve stimulation.

The actions of atropine and quinidine were much less striking than that of pethidine. Atropine sulphate in a concentration of 1.2×10^{-4} increased the effect of indirect stimulation by 12 per cent, and increased the effect of direct stimulation by 14 per cent. After full curarization, the same concentration increased the effect of direct stimulation by 16 per cent, at the end of which full curarization was still present. Atropine sulphate in larger concentration

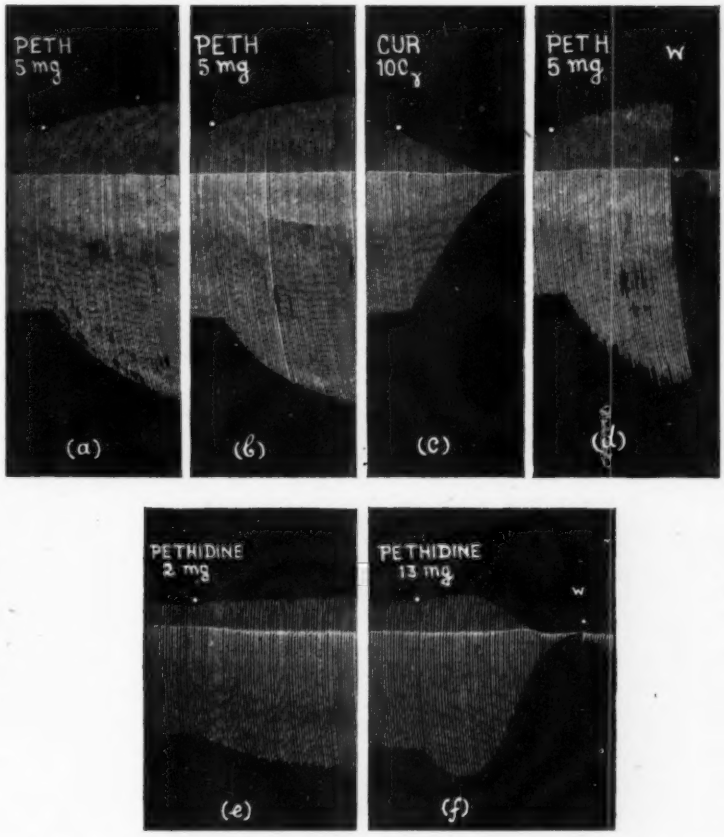

FIG. 2.—Rat phrenic nerve diaphragm preparation in Tyrode, 37° C., 50 ml. bath. *Upper tracing :* single, maximal shock, 7 per minute, 0.7 millisecond. Tyrode solution was changed between (*a*) and (*b*) ; (*b*) and (*c*). (*a*) Indirect stimulation : 5 mg. pethidine hydrochloride increased the muscle contractions. (*b*) Direct stimulation : 5 mg. pethidine had the same effect as before. (*c*) Indirect stimulation : progressive paralysis of the muscle after 100 μg. *d*-tubocurarine chloride. (*d*) Direct stimulation : 5 mg. pethidine still produced increased muscle tension. Just before wash-out (W) indirect stimulation was applied, but the muscle gave no response. The contractions began to return after washing out the tubocurarine as shown in the right-hand side of the record. *Lower tracing :* single, maximal shock, 7 per minute, 0.5 millisecond delivered through the nerve. Effect of pethidine hydrochloride 2 mg. (*e*) and 13 mg. (*f*). Tyrode solution was changed between (*e*) and (*f*).

(3.2×10^{-4}) caused augmentation of the contractions followed by depression, as shown for pethidine in Fig. 2 (*f*).

Similar results were obtained for quinidine in concentrations $\frac{1}{8}$ and $\frac{1}{4}$ of those of atropine. With procaine the augmentor action was less easily observed, for the depressor action was more powerful; both, however, were recorded, the former when a concentration of 10^{-5} was present in the bath, and the latter when higher concentrations were used.

Antihistamine action on bronchioles

The antihistamine action was observed by the method described by Konzett and Rössler (1940). Guinea-pigs were used, anaesthetized with urethane (1.8 g. per kg.) injected into the peritoneal cavity, the lungs being ventilated by a small Starling respiration pump driving air into the trachea. The excess air which did not enter the trachea operated a lever which recorded a vertical line on a kymograph. The lever rose during the period of insufflation and fell back to zero when insufflation ended. When the bronchioles constricted, the lever rose higher, so that the length of the vertical line was a measure of the constriction. Injections of substances affecting the bronchioles were made through a cannula into the jugular vein.

TABLE II

ANTIHISTAMINE ACTION ON BRONCHIOLES

Bronchoconstriction measured by rise of lever in mm.

Dose (μg.) histamine acid phosphate	Substance	Dose mg.	Bronchoconstriction		Percentage inhibition
			Before	After	
2	Antistin hydrochloride	0.005	34	9	73
2	Atropine sulphate	0.05	45	6	86
12	,,	0.075	33	2	94
2	Benadryl	0.002	42	13	69
2	,,	0.002	39	13	67
4	,,	0.008	28	7	75
4	,,	0.004	15	6	60
2	Pethidine hydrochloride	0.05	27	7	74
5	,,	0.1	28	5	82
2	Procaine hydrochloride	1.0	38	12	68
4	,,	0.5	31	7	77
2	Quinidine hydrochloride	2.0	35	15	57

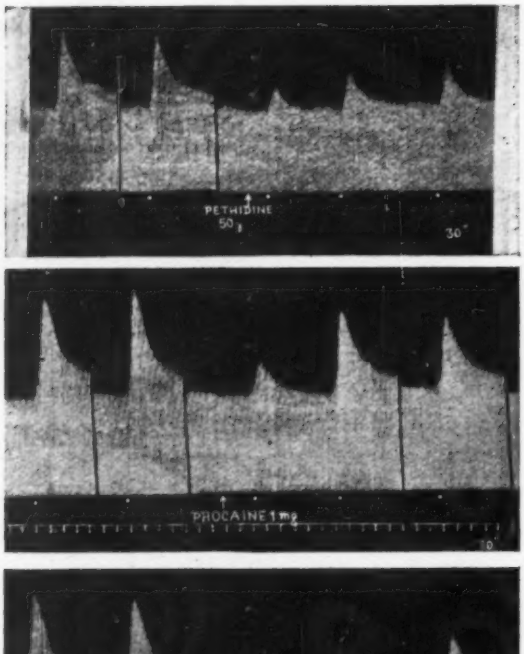

FIG. 3.—Records of the bronchoconstriction produced in the guinea-pig by the intravenous injection of 2 μg. histamine acid phosphate at each white spot. The first two successive injections were made at 8-min. intervals. The effect of histamine was diminished by the intravenous injection of 0.05 mg. pethidine hydrochloride (upper tracing), 1 mg. procaine hydrochloride (middle tracing), and 2 mg. quinidine hydrochloride (lower tracing). Interruption of the records indicates where the drum was stopped.

Examples of the results obtained are given in Fig. 3, which shows the antihistamine action of pethidine, procaine, and quinidine. Histamine acid phosphate was injected at each white spot below the record. The bronchoconstriction produced by at least two successive injections was recorded, the injections being made at 8-minute intervals. Before the third injection, shown in each part of Fig. 3, pethidine or another substance was given, and the effect of histamine was thereby diminished as shown. The results are summarized in Table II. In the table results with two recognized antihistamine substances, "Antistin" (Ciba) and "Benadryl" (Parke, Davis, and Co.), are included in order that a better judgment may be formed of the potency of the other substances. Atropine and pethidine have about one-tenth the potency of antistin, procaine has about one-hundredth the potency of antistin, and quinidine has about one four-hundredth the potency of antistin.

DISCUSSION

The observations provide further evidence that no sharp line of distinction is possible between the actions of atropine, pethidine, procaine, and quinidine, although these four substances are classified quite differently for medical purposes. All of them depress the response of the perfused cervical ganglion to preganglionic stimulation, acting in this way like *d*-tubocurarine.

The powerful action of atropine in blocking the preganglionic stimulation in the perfused superior cervical ganglion is contrary to the general belief that the nicotine-like action of acetylcholine is not influenced by atropine. Abdon (1940) has shown that the difference in the antagonism of atropine to nicotine-like and muscarine-like actions of acetylcholine is quantitative rather than qualitative.

The actions of atropine, procaine, pethidine, and quinidine on the skeletal muscle (diaphragm) of the rat are complex. In small concentrations these substances increase the contraction to a single maximal stimulus, but in higher doses they exert a curare-like effect; procaine, pethidine, and quinidine depress the muscle in concentrations much less than that required for atropine. The augmentation of the muscular twitches in response to direct stimulation by any one of these substances was not affected by *d*-tubocurarine: therefore, the point of attack was directly on the muscle itself. Procaine acts on skeletal muscle at several points; it interferes with the liberation of acetylcholine at the motor nerve endings (Harvey, 1939a), depresses the motor endplate, and also acts directly on the muscle (Kubota and Macht, 1919; Macgregor, 1939). Harvey (1939b) showed that quinine exerts its action on the motor endplates as well as on the muscle itself. Quinidine, a stereoisomer of quinine, probably acts in the same way. Like tubocurarine, atropine raises the threshold of the skeletal muscle to stimulation, and it has been suggested that it prevents the action of acetylcholine at the nerve terminals (Brown, 1937; Bülbring, 1946). Since these substances block ganglionic transmission, it may be that they interfere with the action of acetylcholine at the motor nerve terminals of the skeletal muscle.

Loew, Kaiser, and Moore (1946) compared the relative potency of benadryl, pethidine, and atropine in reducing the mortality rate of guinea-pigs exposed

to histamine aerosol. They found that pethidine had about a quarter of the activity of benadryl, but it was three times more potent than atropine. Using a similar technique, Graham (1947) showed the superiority of benadryl over antistin. Frommel, Favre, and Vallette (1947) had to use a large amount of procaine to protect their guinea-pigs from the effect of histamine given in the form of spray. Thus a fair amount of correlation exists between the results obtained by the above investigators and the findings in the present experiments, though the modes of administration of histamine were different. It is remarkable that quinidine also possesses some antihistamine activity.

Summary

Atropine, pethidine (demerol), procaine, and quinidine have the following properties in common:

(1) When injected into the fluid perfusing the superior cervical ganglion they depress the contraction of the nictitating membrane in response to preganglionic stimulation.

(2) They augment the contractions of the isolated rat diaphragm, both when it is stimulated through the nerve and when it is stimulated directly after curarization. Very high concentrations depress the contractions.

(3) They depress the bronchoconstrictor action of histamine in the guinea-pig.

I am most deeply indebted to Professor J. H. Burn for the guidance and encouragement which he has given throughout and for the facilities provided.

The work was carried out during the tenure of a fellowship from the Government of India.

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LIBERATION OF *NOR*ADRENALINE FROM THE SUPRARENAL GLAND

BY

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(Received March 30, 1949)

The denervated nictitating membrane of the cat has frequently been used to detect the liberation of the humoral transmitter during stimulation of sympathetic nerves, because it was found to be more sensitive than the normal membrane (Cannon and Rosenblueth, 1933; Greer, Pinkston, Baxter, and Brannon, 1938; Gaddum and Goodwin, 1947). In a previous paper (Bülbring and Burn, 1938) we have studied the difference in the response of the normal and the denervated membrane to various sympathomimetic substances. We found that there were many exceptions to the rule that the denervated membrane is always more sensitive, and indeed for one and the same substance the relative sensitivity varies according to the dose injected. In a film demonstrating the release of Cannon's "Sympathin" by splanchnic stimulation, which we prepared for teaching purposes and which was shown at the International Physiological Congress, 1947, the responses of both the normal and denervated membranes were recorded. Fig. 1 shows such an experiment. The effect of splanchnic stimulation (Fig. 1*b*) on the membranes was different from that of the adrenaline injected in Fig. 1*a*, though the effect on the blood pressure was the same. Stimulation caused a larger contraction of the denervated, and a smaller contraction of the normal membrane. After removal of the suprarenals (Fig. 1*c*), stimulation caused contraction of the denervated membrane, but failed to affect the normal membrane, although the rise of blood pressure was again similar.

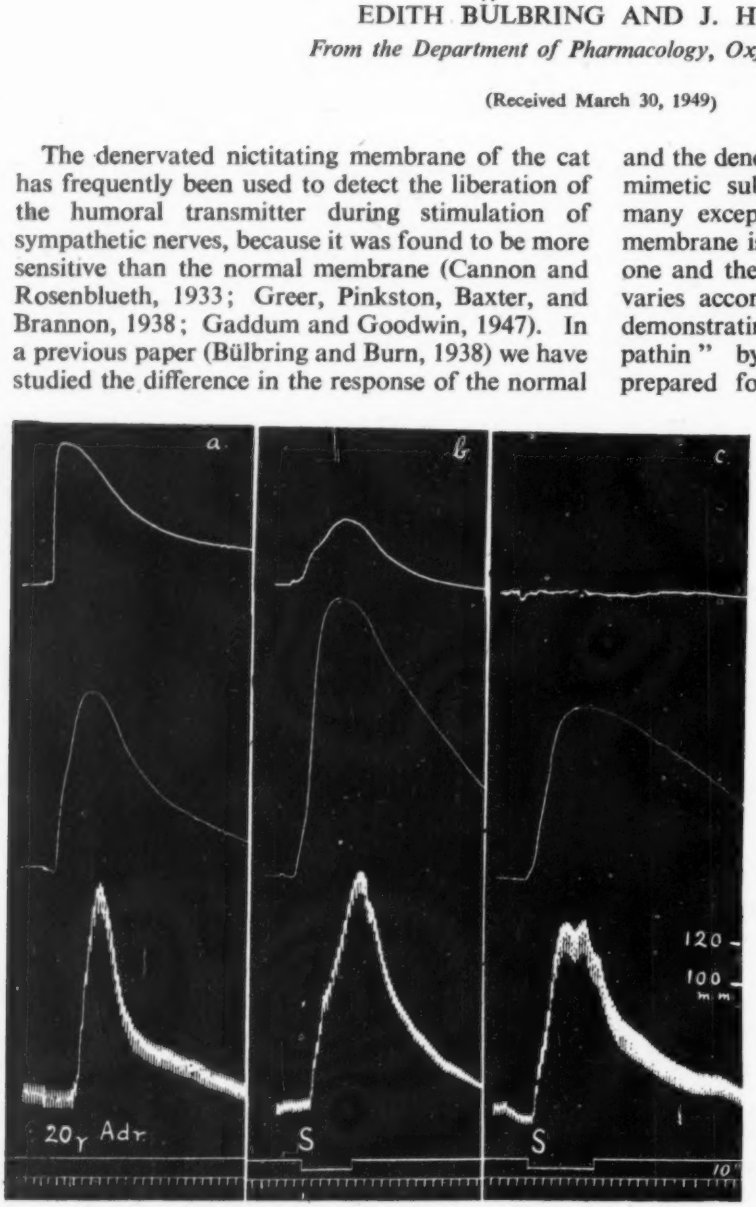

FIG. 1.—Spinal cat. Records from top downwards: normal nictitating membrane, denervated nictitating membrane, blood pressure. Effect of (*a*) 20 μg. adrenaline; (*b*) splanchnic stimulation before and (*c*) after adrenalectomy.

It was shown by Gaddum, Jang, and Kwiatkowski (1939) and also by Gaddum and Kwiatkowski (1939) that the substance liberated by stimulation of the sympathetic nerves to the blood vessels of the perfused rabbit's ear was adrenaline, since it gave the same match with adrenaline in pharmacological and colorimetric tests. We therefore supposed that the differences shown in Fig.1 between the action of adrenaline and that of stimulation were

most probably explained by the rate of entry of adrenaline into the circulation. It seemed possible that a sudden high concentration might stimulate the normal membrane as in (*a*), whereas a slowly rising concentration might stimulate less as in (*b*).

There was another possibility, however, that the differences between the effects of adrenaline and of splanchnic stimulation were to be explained by the release of another substance in addition to adrenaline. Bacq (1933) first suggested that liver sympathin might be *nor*adrenaline. Experimental evidence for this view was obtained by Greer, Pinkston, Baxter, and Brannon (1938), who compared the effect of hepatic nerve stimulation directly with that of *nor*adrenaline and adrenaline. In 1946 von Euler obtained evidence for the presence of *nor*adrenaline in sympathetic nerves, and in the following year Gaddum and Goodwin found that the substance released by hepatic nerve stimulation was probably *nor*adrenaline or possibly tyramine. In 1948 von Euler identified *l-nor*adrenaline in the splenic nerves of cattle, and Schmiterlöw similarly found it present in extracts from arteries. The foregoing observations suggested that the result shown in Fig. 1*c* might be due to the liberation of *nor*adrenaline, and the result in Fig. 1*b* to a mixture of adrenaline coming from the suprarenal glands, with *nor*adrenaline coming from sympathetic nerve endings.

The first indication that *nor*adrenaline might also be present in the suprarenal gland was given by Holton (1948), who found large amounts of it in suprarenal tumours of the type phaeochromocytoma. So far as the normal gland is concerned, observations by Meier and Bein (1948) led them to conclude that it released some other substances in addition to adrenaline. They found that a small dose of adrenaline, which caused an increased blood flow in the femoral artery, caused a decrease in flow after adrenalectomy; the normal response could be restored by slow infusion of *nor*adrenaline. Schümann (1948) has recently published a preliminary account of experiments in which he found that an extract of pig's suprarenal gland had a smaller hyperglycaemic action in the rabbit than an equipressor amount of adrenaline, but a similar hyperglycaemic action to a mixture of equal parts of adrenaline and *nor*adrenaline.

Our own experiments, to be described below, were designed to discover what was released by the normal gland during splanchnic stimulation. A brief note on this work has already appeared (Bülbring and Burn, 1949).

Methods

Cats were prepared by the removal of the right superior cervical ganglion in an aseptic operation carried out 1–2 weeks before. On the day of experiment anaesthesia was induced by ethyl chloride followed by ether, and a spinal preparation was made cutting the cord at the 2nd cervical vertebra and destroying the brain. The viscera were removed and the vessels to the left kidney were tied. In some experiments the vessels to both kidneys were tied. The left splanchnic nerve was prepared for stimulation with platinum electrodes embedded in perspex. Stimulation was applied for 30 sec. from an induction coil, the primary circuit being interrupted by Lewis's rotary contact breaker, at a rate of 20 per sec. The blood pressure was recorded from the femoral artery, and the contractions of both nictitating membranes were recorded by isotonic levers of the same weight and magnification. All substances were given by intravenous infusion into a femoral vein during a constant period of 30 sec., this being the same as the duration of stimulation. The sample of *nor*adrenaline was the racemic compound kindly given to us by Dr. M. L. Tainter of the Sterling-Winthrop Research Institute.

Results

Adrenaline, nor*adrenaline, and stimulation*

When the effects of adrenaline and *nor*adrenaline were compared with that of splanchnic stimulation the doses were chosen to give the same rise of blood pressure. It was found that in the eviscerated animal the pressor action of *nor*adrenaline was not stronger than that of adrenaline, as it is before evisceration, but was either equal to it or weaker. Thus in Fig. 2 the effect of 10 μg. *dl-nor*adrenaline is approximately the same as that of 5 μg. *l*-adrenaline, while in the experiment from which Fig. 3 was taken 15 μg. *dl-nor*adrenaline was equal to 5 μg. *l*-adrenaline.

The nictitating membranes reacted differently to equipressor doses. The sensitivity of the normal membrane was much greater to adrenaline than to *nor*adrenaline, while the denervated membrane was very sensitive to both. The effect of splanchnic stimulation on the normal membrane was intermediate between the two. In Fig. 2 stimulation produced similar effects on the blood pressure and denervated membrane to those produced by 10 μg. *dl-nor*adrenaline and 5 μg. adrenaline, while on the normal membrane its effect was very much greater than that of *nor*adrenaline but less than that of adrenaline. In Fig. 3, on the other hand, the effect of stimulation on the normal membrane was much closer to that of *nor*adrenaline; adrenaline produced a greater effect than either, in spite of the fact that the dose of adrenaline was too small to be equipressor and to produce the same contraction on the denervated membrane.

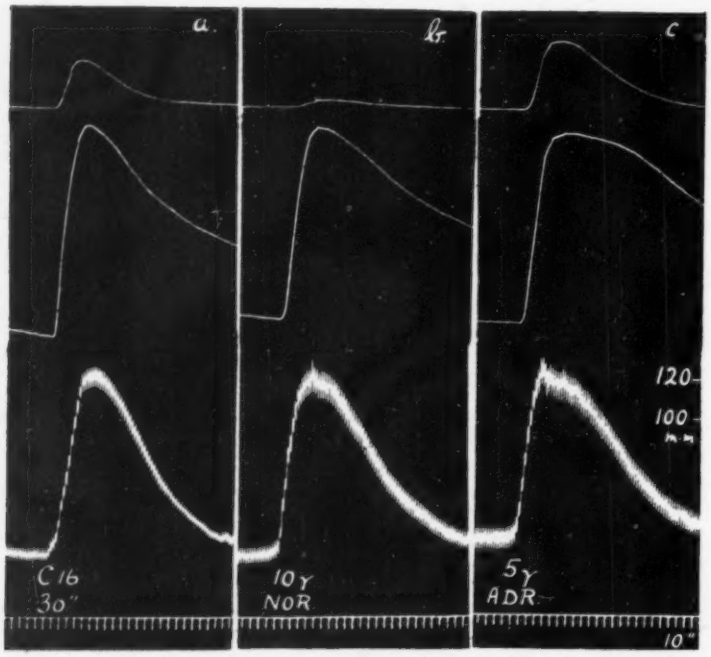

FIG. 2.—Spinal eviscerated cat. Records as in Fig. 1. Note that the response of the nictitating membranes to splanchnic stimulation (*a*) is intermediate between that to 10 μg. *dl-nor*adrenaline (*b*) and that to 5 μg. *l*-adrenaline (*c*).

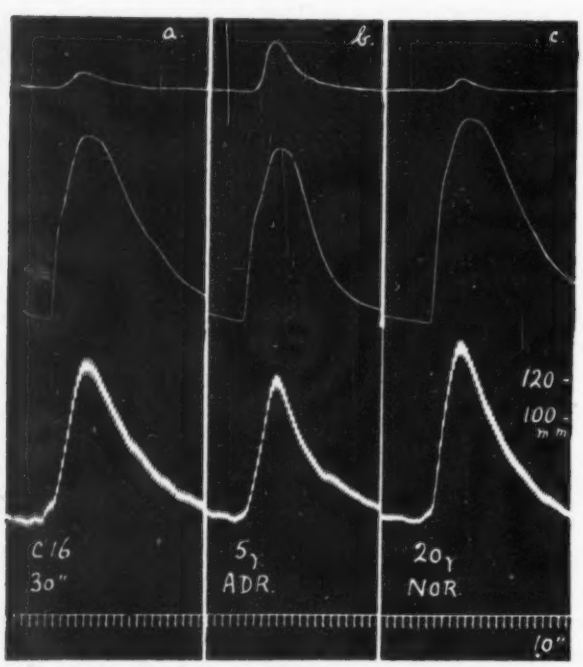

FIG. 3.—Spinal eviscerated cat. Records as in Fig. 1. Note that the response of the nictitating membranes to splanchnic stimulation (*a*) is very similar to that caused by 20 μg. *dl-nor*adrenaline (*c*) but not to that caused by 5 μg. *l*-adrenaline (*b*).

Ratio of the size of contractions

In twelve experiments we have compared the sensitiveness of the denervated with that of the normal membrane by calculating the ratio of the size of their contractions. The results are given in Table I. If the ratios for adrenaline in the different experiments are examined it will be seen that there are five exceptions to the rule that the denervated membrane is more sensitive. In Expts. 6, 7, and 10 the ratio is less than 1.0, and in Expts. 8 and 11 it is equal to 1.0; this may be due to the use of small doses only. The ratios for *nor*adrenaline are always greater than 1.0, as are those for stimulation.

The main point of Table I is that in each experiment the ratio for *nor*-adrenaline is greater than that for adrenaline, and that the ratio for stimulation is intermediate. Each ratio given in the Table is the mean of all the determinations made in that particular experiment. It is true that the ratio for any substance depends on the amount injected; thus in one experiment the ratio for 5 μg. adrenaline was 2.3, for 10 μg. it was 2.1, and for 20 μg. it was 1.85; but since the range of doses used to match splanchnic stimulation was narrow the effect of dose on ratio was negligible for our purposes.

TABLE I

RATIO OF CONTRACTIONS OF DENERVATED TO NORMAL MEMBRANE

Experiment	Adrenaline	Stimulation	*Nor*adrenaline
1	1.9	2.05	7.8
2	2.8	6.1	17.5
3	3.0	5.3	23.0
4	1.5	2.5	9.2
5	1.4	4.4	6.5
6	0.9	1.36	6.1
7	0.8	2.5	7.2
8	1.0	1.2	1.8
9	1.4	1.8	6.2
10	0.65	1.04	1.16
11	1.0	1.16	2.1
12	1.14	1.51	5.2

Matching the effect of stimulation

We were able to match the effect of stimulation on the nictitating membranes by infusing mixtures containing different proportions of adrenaline and

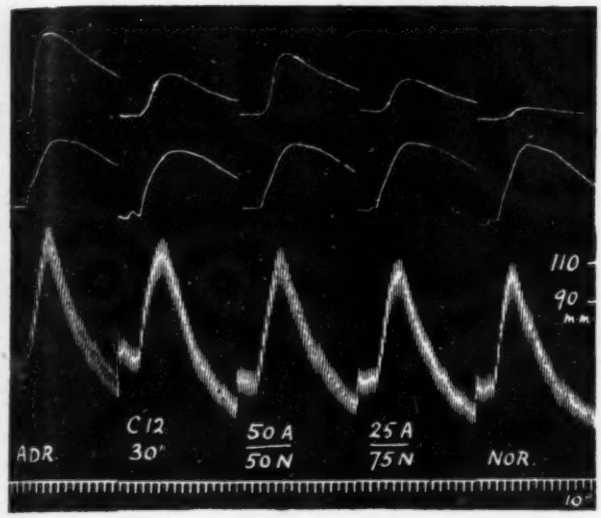

FIG. 4.—Spinal eviscerated cat. Records as in Fig. 1. Estimation of the proportion of adrenaline and *nor*adrenaline liberated by splanchnic stimulation (see text).

*nor*adrenaline. The solutions used were always 1.25 μg. adrenaline per c.c. and 2.5 μg. *dl-nor*adrenaline per c.c. A mixture described as 75 per cent adrenaline and 25 per cent *nor*adrenaline contained three parts by volume of the first and one part of the second, etc. The total amount of any mixture infused was adjusted to produce a rise of blood pressure equal to that of an adjacent stimulation. The mixture which also produced the same effect on the nictitating membranes both in size and in ratio was taken as a match. Fig. 4 shows the procedure. The effect of adrenaline was first recorded, followed by that of stimulation. From the response of the nictitating membranes it was obvious that stimulation did not release adrenaline only. A mixture was then infused of 50 per cent adrenaline and 50 per cent *nor*adrenaline. The effect on the membranes indicated that this mixture contained a larger proportion of adrenaline than was released by stimulation. Infusion of a mixture containing 25 per cent adrenaline and 75 per cent *nor*adrenaline produced a response which indicated that this mixture contained too small a proportion of adrenaline.

Estimation of proportion

The exact figure for the proportion of adrenaline and *nor*adrenaline was determined from a curve relating the ratios of the contractions to the mixtures infused in each experiment. Curves for three experiments are reproduced in Fig. 5. When the mean ratio for stimulation was found, reference to the curve gave the proportions of adrenaline and *nor*adrenaline released. In the three experiments represented in Fig. 5, the proportions were 55, 45, and 20 per cent adrenaline respectively.

A calculation was also made of the proportion of adrenaline liberated by successive stimulations in each experiment. The results are shown in Table II

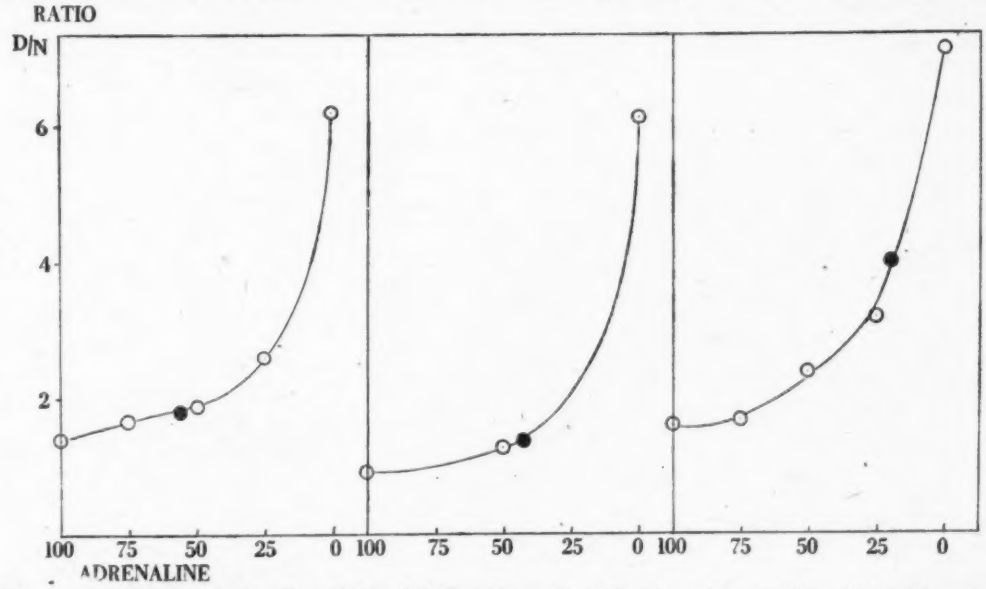

FIG. 5.—Three experiments showing the relation between the ratio of the size of contraction of the denervated to that of the normal nictitating membrane (ordinates) and the percentage of adrenaline present in the mixtures with *nor*adrenaline infused (abscissae). The black circles represent the mean ratio obtained by splanchnic stimulation in each experiment.

TABLE II

PERCENTAGE OF ADRENALINE RELEASED BY SUCCEEDING STIMULATIONS

Experiment	Stimulations 1st	2nd	3rd	4th	5th	Mean
4	98	95	86	82	42	81
5	65	75	50	10	0	40
6	80	55	40	32	37	49
7	·15	12	32	34	25	24
8	·32	28	32			31
9	·40	67	85	47		59
10	·35	42	35	27	22	30
11	70	42	37	38		47
12	95	95	35	45		67
Mean ..	59	57	48	39	25	47

in which it will be seen that in five experiments, in which initially the proportion was high, it fell steadily as the experiment proceeded. In the four experiments in which the initial proportion was lower, there was little change except in Expt. 9. The mean results for successive stimulations shown at the bottom of Table II clearly indicate a steady fall in the proportion of adrenaline released.

Experiments with other substances

While the experiments so far described gave results compatible with the assumption that splanchnic stimulation liberated a mixture of adrenaline and *nor*adrenaline, they did not exclude the possibility of other substances, such as *l*-dihydroxy*nor*ephedrine (corbasil), epinine, and hydroxytyramine, being present. We therefore investigated these substances. When their pressor effects were examined in the eviscerated spinal animal, it was found that the ratios of equipressor doses, taking *l*-adrenaline = 1, were *l*-dihydroxy*nor*ephedrine = 2, epinine = 17, and hydroxytyramine = 50. From these figures it follows that if epinine or hydroxytyramine is contributing a large part of the pressor effect of splanchnic stimulation, very large amounts must be released. Furthermore, when these two substances were injected they depressed the effect of a succeeding stimulation or injection of adrenaline. For this reason we did not consider them further. Dihydroxy-*nor*ephedrine remained. With this substance mixtures containing adrenaline could be prepared to give a curve similar to those in Fig. 5. The ratios obtained in three experiments are given in Table III.

Inspection of Table III will show how small was the change in the ratio as increasing proportions of corbasil were introduced into the mixture. The difference in the curves for adrenaline *plus nor*-adrenaline and for adrenaline *plus* corbasil is

TABLE III

RATIO OF CONTRACTIONS OF DENERVATED TO NORMAL MEMBRANE WITH MIXTURES OF ADRENALINE (A) AND OF *l*-CORBASIL (C)

Experiment	Adren. 100%	A75% C25%	A50% C50%	A25% C75%	Corb. 100%	Stim.
13	0.65		0.74	0.76	1.25	1.04
14	1.0	1.02	0.98		1.86	1.16
15	1.14	1.25	1.25	1.4	2.4	1.51

illustrated in Fig. 6, which shows that the effect of stimulation in that experiment could be matched with either of these combinations, but the matching mixture with corbasil would contain only 10 per cent adrenaline. Corbasil is, moreover, excluded when the actual membrane contractions are considered as shown in Fig. 7. A mixture containing 25 per cent adrenaline and 75 per cent corbasil, which was equipressor to stimulation, produced contractions

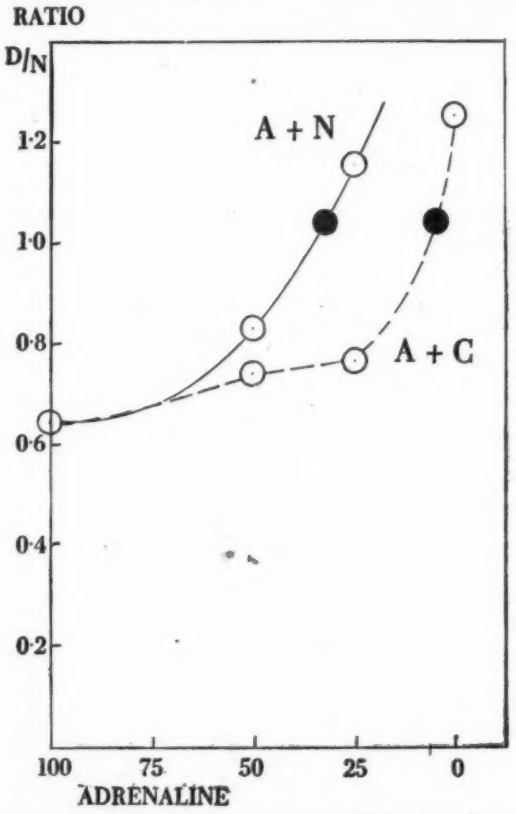

FIG. 6.—Curves relating the ratio of the contractions of the two nictitating membranes (ordinates) to the proportion of adrenaline (abscissae) present in mixtures containing *nor*adrenaline (continuous line, A + N) and corbasil (broken line, A + C). The ratio for splanchnic stimulation (black circles) may be found on both curves.

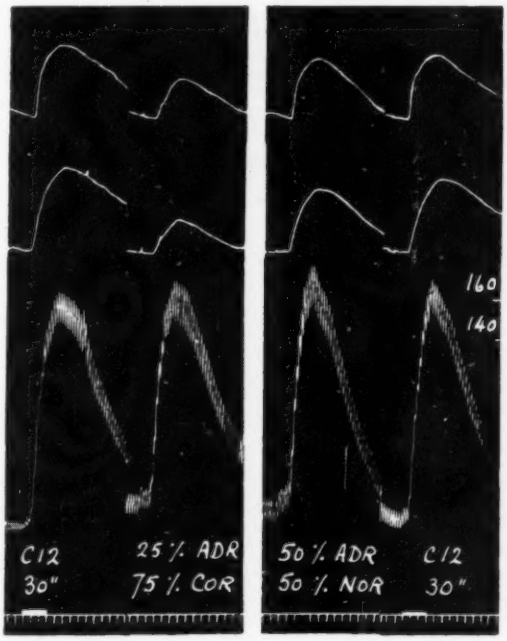

FIG. 7.—The ratio of the contractions produced by stimulation is very similar to that produced by a mixture of adrenaline and corbasil, but the actual size of the contractions is seen to be different. This is not so when stimulation is compared with the mixture of adrenaline and *nor*adrenaline.

much smaller than those caused by stimulation, and a higher proportion of corbasil, though matching the ratio, would have produced still smaller contractions. A larger amount of this mixture would have matched both the ratio and the actual size of contractions, but would have produced too great a rise of blood pressure. When stimulation was compared with adrenaline *plus nor*adrenaline, the blood pressure, the ratio, and the actual size of contractions could all be matched.

Cats fed with methionine

With the possibility in mind that the release of *nor*adrenaline from the suprarenal gland might be due to deficient methylation of this substance, we fed a series of cats on a diet containing 0.2 g. *dl*-methionine daily for 6–10 days. Results which may be compared with those in Table II are shown in Table IV. The cats not only received methionine but were liberally supplied with kipper and other fish in order to encourage them to eat well. The figures in Table IV show that this treatment did not suffice to change the substance released by splanchnic stimulation to adrenaline only. The mean figures are slightly higher than those in Table II and the decline in the proportion of adrenaline released

TABLE IV

METHIONINE-FED CATS. PERCENTAGE OF ADRENALINE RELEASED BY SUCCEEDING STIMULATIONS

Experiment	No. of days fed	Stimulations 1st	2nd	3rd	4th	5th	Mean
16	7	92	95	96	97		95
17	7	57	57	45			53
18	10	70	67	100	65	53	70
19	9	33	30	45			36
20	10	75	78	78	95	100	85
21	6	42	44	43	43	35	41
22	7	77	66	43			62
	Mean	64	62	64	75	62	63

with succeeding stimulation is absent. In two of the experiments (Nos. 16 and 21) the condition of the cats was particularly good; in the first the proportion of adrenaline was almost 100 per cent; in the second, however, it was consistently below 50 per cent. We intend to investigate the effect of diet in a further series of cats.

DISCUSSION

During the last 12 years there has been a growing body of evidence that *nor*adrenaline is liberated as a sympathetic transmitter. Recent work of von Euler (1948) has established its presence in nerve extracts, and Peart (1949) has demonstrated its liberation. The evidence does not necessarily exclude adrenaline as another sympathetic transmitter and is, therefore, not in conflict with the findings of Gaddum and Kwiatkowski (1939). Till the last few months there has been no suggestion that the suprarenal medulla liberated anything but adrenaline. The chemical isolation of this substance nearly 50 years ago appears to have inhibited inquiry into the question. It is, however, a more important point than the identity of the transmitter, since while the latter has in the main only a local effect, substances liberated from the suprarenal medulla have a general action throughout the body. One difficulty in the way of this investigation has been the lack of suitable methods of distinguishing between adrenaline and *nor*adrenaline. The finding by West (1947) of the specificity of the rat uterus method for adrenaline has, however, proved a very useful advance. We have observed that the difference in the sensitiveness of the normal and denervated nictitating membranes provides a convenient test to distinguish the two substances in the blood, and have been able to show that splanchnic stimulation in the cat liberates both substances from the suprarenal gland. In the spinal cat the proportion of

adrenaline liberated is about 60 per cent, and the evidence is strongly in favour of the remaining 40 per cent being *nor*adrenaline. When stimulation is repeated the proportion of adrenaline steadily declines, and this observation suggests that *nor*-adrenaline is not only liberated but is a precursor of adrenaline in the gland. Holtz and Kroneberg (1948) have proposed epinine rather than *nor*-adrenaline as the precursor, on the ground that the latter substance, occurring naturally in the body—in the urine (Holtz, Credner, and Kroneberg, 1944–7), in sympathetic nerves (v. Euler, 1948), and as a constituent of the suprarenal medulla (Schümann, 1948)—is likely to be an end product and not a precursor.

A different view has been taken by Goldenberg *et al.* (1948). They compared the effect of intravenous infusion of adrenaline and *nor*adrenaline in a series of patients and found that the former produced a decrease of peripheral resistance and the latter an increase. They suggest that *nor*adrenaline is a precursor of adrenaline, and that essential hypertension results from a failure to effect methylation. We have tested the hypothesis of deficient methylation by feeding a series of cats on methionine, and have not obtained more than a slight and not significant increase in the proportion of adrenaline liberated from the gland. This proportion, however, was maintained with successive stimulations and did not decline as in other cats.

We think that there is truth in both views. We believe that *nor*adrenaline is a precursor of adrenaline, as suggested by Blaschko (1942), and that in the healthy animal the process of methylation must be sufficiently vigorous to maintain a store of adrenaline which is not exhausted no matter how frequent the splanchnic impulses. If there is deficient methylation then, as Goldenberg *et al.* have suggested, hypertension may follow, and the recent work of Hartroft and Best (1949) showing that choline deficiency causes hypertension in rats may find an explanation along these lines. We believe in addition that *nor*adrenaline is released in the blood to exert a function of its own. Meier and Bein (1948) have shown that the effect of small doses of adrenaline in causing increased blood flow in the femoral artery is reversed after adrenalectomy, but is restored by the infusion of *nor*adrenaline. This suggests that the two substances are released together as is borne out by our own observations.

Summary

1. In the spinal cat the ratio of the contraction of the denervated nictitating membrane to that of the normal membrane is very much greater when *nor*adrenaline is injected than when adrenaline is injected.

2. If the cat is eviscerated and the splanchnic fibres to one suprarenal gland are stimulated the effect on the membranes is intermediate between that of adrenaline and that of *nor*adrenaline and can be matched by infusing a mixture of the two.

3. It is not possible to make such a match with a mixture of adrenaline and dihydroxy*nor*ephedrine, and other reasons exclude epinine or hydroxytyramine as substances which might be released from the suprarenal gland.

4. If the splanchnic is stimulated repeatedly, a gradual decline in the proportion of adrenaline secreted was observed in normal cats, but this decline seemed to be absent in cats fed on a diet rich in methionine.

5. The evidence indicates the release of *nor*-adrenaline as well as of adrenaline from the suprarenal gland, in amounts varying from 20–80 per cent of the total.

We wish to thank Mr. H. W. Ling for his very careful assistance.

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INTRACORNEAL INFECTION AS A METHOD FOR TESTING ANTITUBERCULOUS SUBSTANCES

BY

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(Received April 5, 1949)

Since 1935 several synthetic and antibiotic substances with marked antituberculous activity have been discovered. Many more such substances will be produced in the future, all of which will require screening and comparing with those already available. The *in vitro* screening of these compounds for their antituberculous activity is relatively simple. If these tests give a positive result and the substance is of low toxicity then the vital stage of testing the substance *in vivo* against experimentally produced tuberculous infections in animals is reached. At present guinea-pigs or mice are the experimental animals generally used, and infection is established by the intravenous, intraperitoneal, or subcutaneous route. The efficiency of the substance is measured by comparing the survival times, weight changes, and detailed histopathological findings of the treated group with those of the control group. Feldman and Hinshaw (1945), summarizing their extensive experience with guinea-pigs as the test animal, conclude that at least sixty days are needed for a screening test. Youmans and Raleigh (1948), using mice, sacrificed all survivors at thirty-five days and compared average survival times, weight changes, and detailed histopathology with the controls. Martin (1946) and Hoggarth and Martin (1948) also used mice but in larger groups, and inoculated them with a fairly acute type of infection: they determined the antituberculous activity by a statistical analysis of differences in mean survival time. One other method worth mentioning makes use of the chorio-allantoic membrane of the developing chick embryo; this was originally suggested as a method for screening compounds by Emmart and Smith (1941). Here the chorio-allantoic membrane is inoculated with tubercle bacilli and the drug for screening is introduced into the amniotic cavity or yolk sac. Although this is an *in vivo* method by definition, the natural progress of the tuberculous lesion is so rapid and the environment so specialized that it adds little information to that obtained from an *in vitro* test.

At present it is impossible to decide which of the methods in guinea-pigs and mice is the best as there are so many variables. The guinea-pig is uniformly a highly susceptible animal to tuberculosis and therefore the lesions are of the acute type and unaffected by any natural host resistance. Mice generally have a greater and more variable natural resistance with a marked strain variation and therefore every grade of activity may be met. Whatever the final choice, all the methods at present available for satisfactory *in vivo* screening tests are laborious and time consuming. The assessment is frequently delayed till the animal is sacrificed after many weeks and not till then is it possible to say whether the drug is of value or otherwise. The *in vivo* methods at present are formidable enough to curtail very seriously the screening of potentially active antituberculous substances.

The present paper describes an entirely new *in vivo* method which is relatively simple to perform and gives unique opportunity for observation of the tuberculous lesion, and comparison with a control lesion, throughout the chemotherapeutic trials. The method was tried with proved antituberculous substances—viz., streptomycin and the sodium salt of *para*-aminosalicylic acid, and the results so obtained are also described.

METHODS

All experiments were performed on mature rabbits of various breeds and both sexes. Throughout the experiments a bovine strain of *Mycobacterium tuberculosis* was used to inoculate the cornea because rabbits have a well-developed natural immunity to human strains. The strain, which was the same as that used in earlier similar work (Robson, 1944), was kindly supplied by Dr. W. M. Levinthal of the Royal College of Physicians, Edinburgh. The strain was sensitive to between 0.2 and 0.4 μg. streptomycin per ml. of culture medium—i.e., similar in sensitivity to the standard virulent human strain, H37Rv.

A synthetic medium containing Tween 80 and bovine albumin (Dubos and Davis, 1946), as modified by the Medical Research Council (1948), was used for culturing

the organism. Seven-day actively growing cultures gave a homogeneous suspension which was adjusted, by means of turbidimetric estimation with a photoelectric absorptiometer, to contain 0.1 mg. (dried weight) of organisms per ml. (equivalent to between 10^8 and 10^9 viable organisms per ml.). After many trials it was found that an inoculum containing approximately 300 tubercle bacilli consistently produced a suitable progressive tuberculous lesion of the cornea.

As in earlier work (Robson, 1944), the lesions were produced by intracorneal injection, with a tuberculin syringe with a fine and short bevelled needle, into rabbits deeply anaesthetized with ether. A white bleb was produced in the cornea and, by restricting the bleb to about 5 mm. in diameter (which gives an inoculum volume of about 0.03 ml. of a suitably diluted standardized suspension of organisms), uniformity in size of inoculum and lesion was obtained.

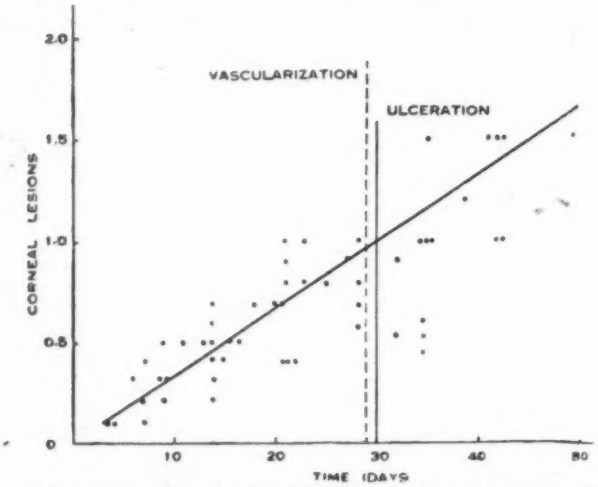

FIG. 1.—Showing the development of tuberculous corneal lesions produced by an inoculum of approximately 300 tubercle bacilli. The average times at which vascularization and ulceration occur are also shown.

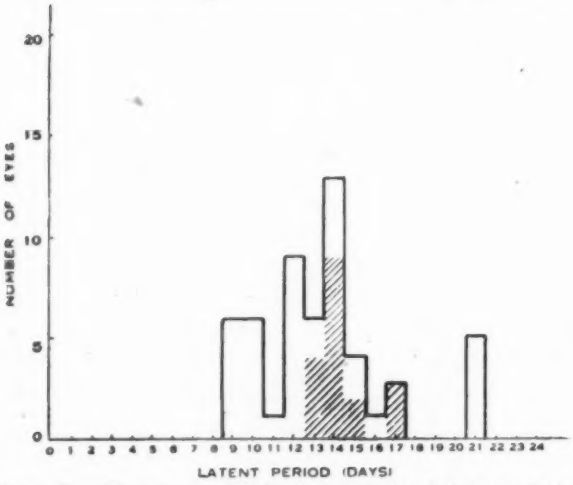

FIG. 2.—Frequency distribution of the latent periods after inoculation of the cornea with tubercle bacilli (analysis of 55 eyes). The shaded area shows the results in one particular experiment.

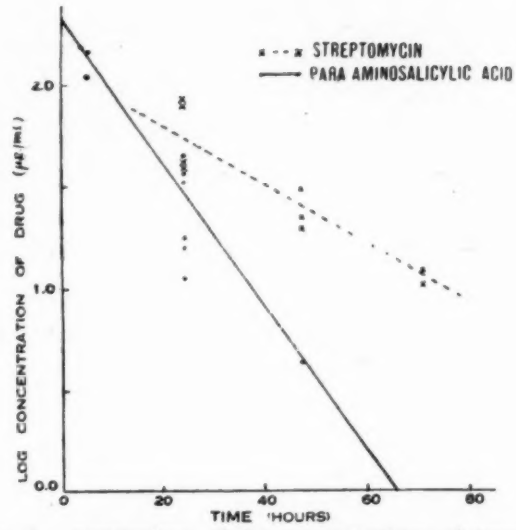

FIG. 3.—Comparison of the concentrations of streptomycin and the sodium salt of *p*-aminosalicylic acid in the aqueous after intravitreous injection of 2 mg. streptomycin and 10 mg. sodium *p*-aminosalicylate.

With this technique lesions are consistently produced. Single or multiple minute white primary lesions appear in the needle track and rapidly progress in size by direct spread and by peripheral satellite tubercle formation. At the same time caseation occurs at the centre of the lesion which later, at about the thirtieth day, breaks down to form an ulcer, and vessels grow in from the limbus (Plates I and II). By an arbitrary numerical method it is possible to study quantitatively the natural development of these corneal lesions and by this method it is found that they progress at a remarkably constant rate (Fig. 1).

The development of a primary lesion is preceded by a latent or incubation period which again has proved to be reasonably constant. About 50 per cent of all the lesions so far observed have appeared within a three-day period—i.e., between the 12th and 14th days inclusive after inoculation. The latent period is even more constant in any one particular experiment, and in one such experiment the lesions appeared within a three-day period in 85 per cent of animals (Fig. 2).

It has been previously shown (Gardiner *et al.*, 1948) that after a single intravitreous injection of 2,000 μg. streptomycin an effective chemotherapeutic concentration is maintained in the ocular fluids for at least three days. (Fig. 3.) The posterior chamber acts as a reservoir for the streptomycin which slowly diffuses into the anterior chamber. No detectable concentration has been found in the opposite untreated eye. It has also been shown that streptomycin produces local toxic effects on the retina and vitreous, but this is not sufficient to interfere with successful antituberculosis therapy. Consideration of the diffusion curve led to the conclusion

that twice-weekly intravitreous injections of 10,000 μg. streptomycin would ensure adequate chemotherapeutic levels throughout the course of therapy. Streptomycin, dissolved in 0.1 ml. of sterile normal saline, was introduced into the vitreous by the method previously described (Duguid *et al.*, 1947).

Experiments with Streptomycin

The experiments with streptomycin in tuberculous corneal infections can be divided into three groups:

A. " Umbrella " type of experiment.

B. Treatment shortly after inoculation.

C. Treatment of developed tuberculous lesions.

A. Streptomycin " umbrella " experiment

In this experiment streptomycin was injected into the vitreous in order to produce a chemotherapeutic level in the cornea, which was then inoculated with tubercle bacilli. Six rabbits were used; on the first day 10,000 μg. streptomycin were injected into the vitreous of the right eyes; 24 hours later the corneae of both eyes of all six rabbits were inoculated with about 300 tubercle bacilli. Twice-weekly intravitreous streptomycin therapy of the right eyes was continued for a period of 28 days only. The six left eyes acted as controls, and typical progressive tuberculous lesions appeared in them after a latent period of between 13 and 15 days.

In four out of the six animals the treated eyes were still clear from lesions after periods of 77, 82, 82, and 99 days, when the animals were finally destroyed owing to generalized infections with some loss of weight. Of the other two right eyes, one remained clear till the 55th day and the other till the 60th day, when definite lesions appeared for the first time and subsequently progressed and were confirmed to be tuberculous in origin by histological examination.

B. Streptomycin therapy after inoculation

In this experiment streptomycin therapy was started three days after inoculation and maintained twice weekly throughout. Seven animals were used and both eyes were inoculated with tubercle bacilli. Three days later the right eyes were treated with 10,000 μg. streptomycin and maintained twice weekly on this therapy. The left eyes acted as controls. In this experiment different doses of tubercle bacilli were used, ranging from 30 to 3,000 organisms per inoculum, and therefore the latent period varied more, as shown in Fig. 4. One of the seven treated eyes had developed no lesion when the animal was killed on the 94th day; the other six eyes all developed lesions after a prolonged latent period but only one progressed; the others remained minute and later healed. Histological examination showed healed lesions free from acid-fast bacilli. Streptomycin therapy was maintained for periods of 91 to 143 days and in two animals terminated six weeks before they were killed. Reactivation did not occur in either animal.

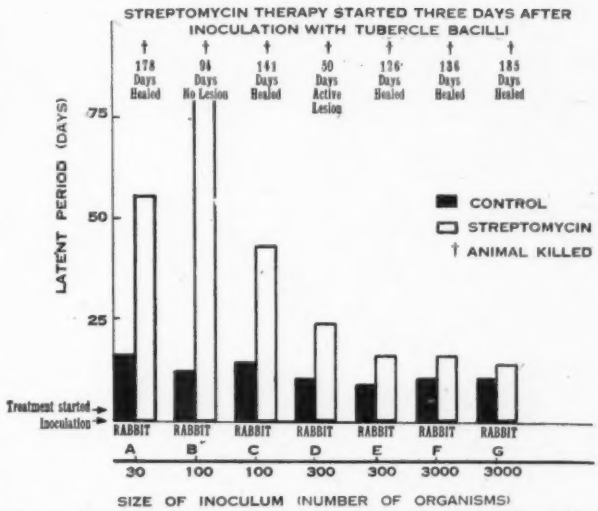

FIG. 4.—Showing effect of continued streptomycin therapy started three days after inoculation of the cornea with different inocula of tubercle bacilli, on the latent period and subsequent lesions.

C. Treatment of developed tuberculous lesions

In this group lesions were allowed to develop before streptomycin therapy was started. Therapy was started at variable times, from the first day of the appearance of a lesion (called L) up to 12 days (L + 12), and continued twice weekly throughout.

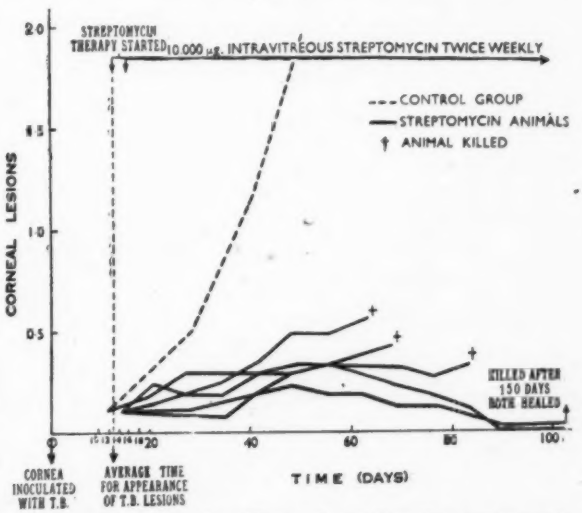

FIG. 5.—Showing the effect of continued streptomycin therapy of developed tuberculous corneal lesions started not more than two days after the first appearance of the lesion.

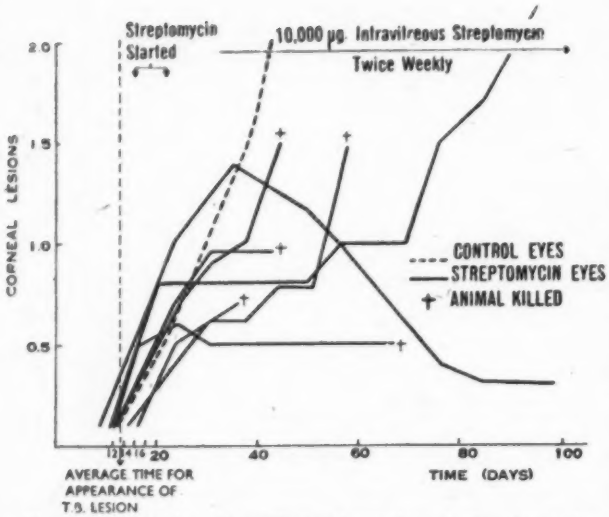

FIG. 6.—Showing the effect of continued streptomycin therapy of developed tuberculous corneal lesions. Treatment was started between the third and twelfth day after the first appearance of the lesions.

This group falls naturally into two parts. In the first, both eyes of five animals were inoculated with about 300 tubercle bacilli, and watched for the development of lesions. Therapy was started on day L to L + 2 in one eye of each animal, the other eye acting as the control. The natural development of the treated lesions was markedly slowed and two out of five showed clinical healing which was confirmed histologically (Fig. 5 and Plates III A and B).

In the second part both eyes of seven animals were inoculated with about 300 tubercle bacilli, and lesions allowed to develop. Treatment was started on day L + 3 to L + 12, the other eye acting as the control. In all treated eyes there was slowing of the natural progress of the lesion as compared with the control eyes, but none showed clinical healing (Fig. 6 and Plates IV A and B). In three eyes treated for 60 to 100 days with streptomycin, cultures of *M. tuberculosis* were obtained from lesions at death. Streptomycin sensitivity tests performed on these showed no increase in resistance to the drug.

EXPERIMENTS WITH *para*-AMINOSALICYLIC ACID

In 1940 Bernheim showed that the oxygen uptake of the tubercle bacillus was stimulated by benzoic and salicylic acid; later Lehmann (1946) discovered that *p*-aminosalicylic acid had considerable bacteriostatic action against the tubercle bacillus *in vitro*. *In vivo* tests with guinea-pigs (Feldman *et al.*, 1947) and clinical trials in man (Erdei, 1948) have given encouraging results. The first step, before proceeding with the rabbit eye *in vivo* test, was to investigate the drug concentration in the ocular fluids after intravitreous injection.

METHOD

Throughout these experiments a sterile solution of 10 g. sodium *p*-aminosalicylate per 100 ml., adjusted to *p*H 6.8, was used in 0.1 ml. quantities for intravitreous therapy.

p-Aminosalicylic acid was estimated on samples of aqueous and vitreous removed from eyes 6, 24 and 48 hours after an intravitreous dose of 10 mg. of the sodium salt. Measured quantities of aqueous and vitreous were treated with 10 per cent (w/v) trichloroacetic acid in order to precipitate proteins and after standing 10 minutes filtered, and the precipitate thoroughly washed with 5 per cent (w/v) trichloroacetic acid. The filtrate was made alkaline and with the addition of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) a yellow-green colour developed which was compared with standard solutions of *p*-aminosalicylic acid, made up in trichloroacetic acid, by means of a Spekker photoelectric colorimeter.

It was shown that after a single intravitreous injection of 10 mg. sodium aminosalicylate a chemotherapeutic level was maintained in the aqueous for at least 48 hours. These results are given in the Table and shown graphically in Fig. 3. Toxic effects were produced in the eye with aminosalicylic acid of similar or even greater severity than with streptomycin but not sufficient to interfere with successful therapy.

TABLE

CONCENTRATION OF *p*-AMINOSALICYLIC ACID AFTER INTRAVITREOUS INJECTION OF 10 MG. OF THE SODIUM SALT

Time after injection hours	Rabbit number	Concentration of aminosalicylic acid (µg. per ml.)	
		Aqueous	Vitreous
6	R. 1 R. eye	144	923
	R. 2 R. eye	232	1233
	R. 3 R. eye	113	1180
24	R. 4 L. eye	16	26
	R. eye	11	45
	R. 5 L. eye	19	—
	R. eye	33	—
	R. 6 L. eye	46	—
	R. eye	37	—
48	R. 7 R. eye	4	6
	R. 8 R. eye	4	6

The bovine strain of *M. tuberculosis* was sensitive *in vitro* to between 0.4 and 0.8 µg. sodium aminosalicylate per ml. To maintain a chemotherapeutic level throughout the experiment 10 mg. sodium aminosalicylate was injected into the vitreous three times a week.

It was anticipated from previous work (Lehmann, 1946) that the effect with aminosalicylic acid would be less definite than that of streptomycin, and therefore an "umbrella" type of experiment was planned to obtain the best possible effect.

para-*Aminosalicylic acid "umbrella" experiment*

In this experiment six rabbits were used; on the first day 10 mg. sodium aminosalicylate were injected into the vitreous of the right eyes; 12 hours later the corneae of both eyes of all rabbits were inoculated with about 300 tubercle bacilli; 10 mg. of sodium aminosalicylate were then injected into the vitreous of the right eyes three times a week for a preliminary period of 28 days. At the end of that period all eyes had developed tuberculous lesions (Fig. 7), but in the treated right eyes the latent

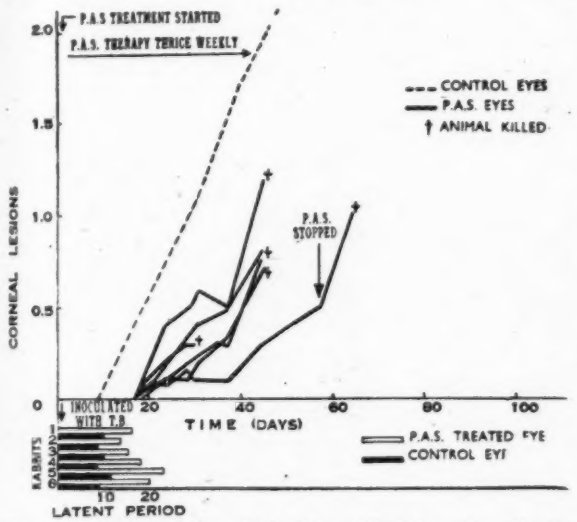

FIG. 7.—Showing the effect of continued aminosalicylic acid (P.A.S.) therapy, started one day before inoculation of the cornea with tubercle bacilli, on the latent period and development of lesions.

period was prolonged by an average of eight days over the left control eyes. Not only was the latent period prolonged in the treated group but the lesions themselves were slightly smaller and less active than the controls. It was decided to continue treatment, and, although at the end of the 38th day there was the same qualitative difference, by the 45th day the treated eyes showed marked deterioration in all but one animal. The remaining one still showed a marked difference and aminosalicylate was continued till the 58th day and the same effect maintained. At that stage the drug was discontinued and the lesion rapidly regressed. Tubercle bacilli isolated from the treated lesions showed no increase in resistance to aminosalicylic acid.

DISCUSSION

The present paper describes a simple method of producing consistently a tuberculous lesion of the cornea in the rabbit. It was found that with a standard inoculum the lesion is preceded by a reasonably constant latent period and, when the development of the lesions is studied quantitatively, a remarkably equal rate of progress is observed. The standard lesion is therefore constant and reliable enough to enable the effect of antituberculous substances to be studied. The results with streptomycin were similar to those reported by other workers—i.e., (1) the natural progress of a developed tuberculous lesion is markedly slowed; (2) the earlier the developed lesion is treated, the greater the chance of complete healing; (3) used as a "screen" or "umbrella," streptomycin is tuberculocidal. The last point was particularly well demonstrated. With *p*-aminosalicylic acid it is possible to demonstrate a favourable effect, such as has been shown in guinea-pigs (Feldman *et al.*, 1947) and in mice (Youmans, 1948), but which has not been confirmed by all workers. The effect is very much less than with streptomycin and confined to slowing of the natural progress rather than actual healing of established lesions.

Streptomycin and *p*-aminosalicylic acid, two substances of known antituberculous activity, both give with this method results comparable with those obtained with other *in vivo* tests, which in general are much more laborious and time consuming. The method has several unique advantages over other methods: the progress of the lesion can be readily observed, thus enabling a lesion of any particular age to be treated or ineffective treatment to be abandoned without any further loss of time. Photographic records of the lesion can be taken as required. The treated and control lesions can be situated in opposite eyes of the same animal, thus allowing for differences in individual animals. In addition, the cornea is suitable for histological or bacteriological examination. Primarily the method is offered as one suitable for preliminary *in vivo* screening of antituberculous compounds with favourable *in vitro* activity—i.e., a method that is capable of giving a more rapid, yet a sufficiently reliable, indication of the *in vivo* activity of some new compound. At the same time a reasonable comparison can be made with other substances of known activity. For such tests few animals and only small quantities of drug (with streptomycin 20 mg. per week per rabbit) are required. Thirty days would be sufficient to give a preliminary indication as to whether a drug was active or inactive against a developed lesion. With *p*-arhinosalicylic

acid the "umbrella" type of test was used which requires even less time. It may well be that the "umbrella" type of screening is the method of choice for the earliest possible indication of antituberculous activity. To what extent the method can be used for more critical evaluation of the effectiveness of drugs depends on further work with other drugs, but present results suggest that it compares not unfavourably with the acute type of screening test in guinea-pigs or mice.

Summary

1. *In vivo* screening tests for antituberculous substances are briefly discussed.

2. Details of a new *in vivo* method making use of the rabbit's eye are given, together with results obtained with streptomycin and *p*-aminosalicylic acid.

3. The advantages of the method are discussed. It is suggested that it may prove of value in the screening of new substances for antituberculous activity *in vivo*.

We are greatly indebted to the Antibiotics Study Section of the U.S. Public Health Service (through Dr. Seger) for the supply of streptomycin; to Herts Pharmaceuticals (through Mr. Seymour) for the supply of *p*-aminosalicylic acid; and to the W. H. Ross Foundation (Scotland) for the Prevention of Blindness, who have defrayed part of the expenses of this work.

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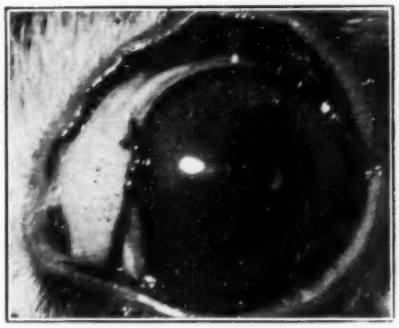
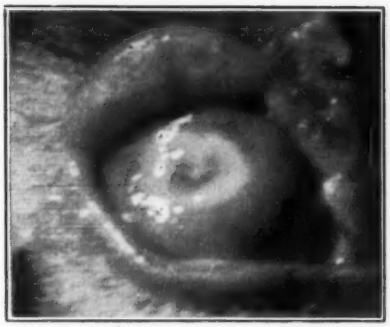
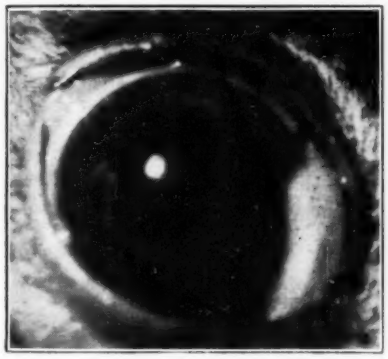


I.—Early tuberculous lesion of cornea (17 days after inoculation).



II.—Advanced tuberculous lesion of cornea, showing caseation with central ulceration (65 days after inoculation).



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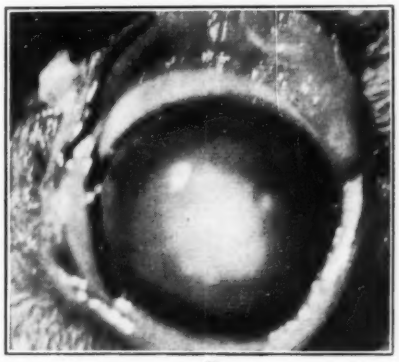
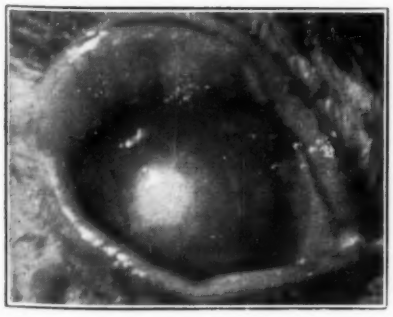


B

III.—Effect of streptomycin therapy started 3 days after first appearance of lesion. Condition of eyes 42 days after inoculation (i.e., 28 days after start of treatment). *A*. Right eye—treated. No lesion visible. *B*. Left eye—control.



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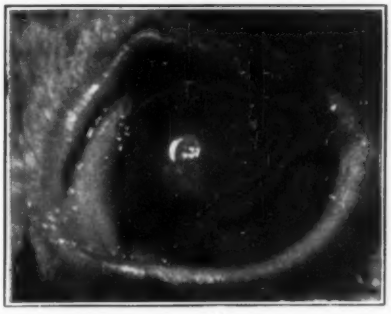

B

IV.—Effect of streptomycin therapy started 6 days after first appearance of lesions. Condition of eyes 38 days after inoculation (i.e., 21 days after start of treatment). *A*. Left eye—treated. Note that lesion has not become dense, especially at the centre where there is probably some attempt at healing. *B*. Right eye—control.

Brit. J. Pharmacol. (1949), **4**, 216.

THE EFFECT OF NEOANTERGAN AND OF BENADRYL ON SERUM-INDUCED MYOCARDITIS IN RABBITS

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(Received March 28, 1949)

Experimental tissue changes can be produced in various tissues of rabbits by the intravenous injection of horse serum. Ehrich *et al.* (1949) have recently reviewed the literature on this subject, adding the confirmatory results of their own investigations. Rich and Gregory (1943a) and McKeown (1947) were of the opinion that the myocardial and valvular lesions so produced were very similar to those found in human rheumatic fever. Other workers suggest that the allergic arteritis seen in the heart and other tissues more closely resembles that of human polyarteritis nodosa. The lesions probably arise from an antigen-antibody reaction in the surrounding tissues, as a result of anaphylactic hypersensitivity. All workers have noted a considerable variation in the incidence of vascular lesions in different experiments. Dammin and Bukantz (1949) observed an incidence of 85 per cent in one experiment and of only 10 per cent in another.

The release of histamine by the antigen-antibody reaction might play a part in producing these lesions, and it was reasonable to investigate whether histamine antagonists could influence their production or severity in serum-sensitized animals.

Kyser *et al.* (1947) recently reported that benadryl and another histamine antagonist (No. 1627) effectively prevented the appearance of rheumatic-like lesions in the myocardium of the serum-sensitized rabbit. In their experiments, complete protection was afforded to sensitized animals by benadryl, but all 11 survivors of a group of 12 sensitized animals not treated with an antihistamine substance developed moderate or severe lesions.

Dammin and Bukantz (1949) have recently reported that benadryl and neohetramine gave no effective protection against the lesions.

Methods

Experimental hypersensitivity was produced by the original technique of Rich and Gregory (1943b). Twenty-two rabbits in all were given intravenous injections of sterile unpreserved horse serum in a dose of 10 ml. per kg. on the 1st and 20th day. The injection of 1 ml serum/kg. 2 days before the second injection of serum was omitted.

Twelve animals were given subcutaneous injections of an antihistamine substance twice daily; six received 5 mg. neoantergan/kg. and six received 10 mg. benadryl/kg. in each dose.

Of these twenty-two animals only one (from the control untreated group) died during the second serum injection. Several others showed marked weakness after this second injection, but all rapidly regained normal activity.

Electrocardiographic records were taken from the benadryl-treated rabbits and their controls by means of a Sanborn Viso-cardiette Electrocardiograph. Records were taken before the first injection and on the 28th day. All animals were killed on the 28th day and the excised hearts fixed in formol-saline for histological examination. The heart sections were stained in the usual way with haematoxylin and eosin, and examined for the lesions detailed below, particular attention being given to the extent and degree of perivascular damage.

Results

In the control group of untreated, sensitized rabbits, 8 out of 10 showed moderate to severe damage characterized by patchy or generalized arteritis with medial necrosis and a periarterial reaction in the adventitia with polymorphonuclear and eosinophil infiltration. Small nodules closely resembling Aschoff bodies were occasionally found, often but not always in relation to vessels showing a severe arteritis. These nodules contained multinucleated giant cells, lymphocytes, eosinophils and large cells with clear cytoplasm like Anitschkow myocytes. Valvular and endocardial lesions were occasionally seen in some animals. Commonly there were also intermuscular collections of mononuclear cells with some oedema and necrotic collagen tissue near the severely affected vessels. The reaction appeared to be mainly a severe arteritis, the occasional endocardial and valvular lesions and Aschoff-like bodies being similar to the lesions of rheumatic carditis.

FIG. 1.—Myocardium of untreated serum-sensitized rabbit. L.P. (mag. × 60). Shows typical severe vascular and perivascular lesions.

Fig. 1 illustrates a typical severe periarterial lesion in a control animal which can be compared with the appearances of a normal rabbit myocardium shown in Fig. 2. An attempt was made to give some quantitative indication of the severity of the disease in each rabbit. A points system was devised, marks being awarded for intermuscular and perivascular infiltration, Aschoff-like bodies, muscle and collagen degeneration. Two observers assessed each animal independently. Normal rabbit sections were available for comparison.

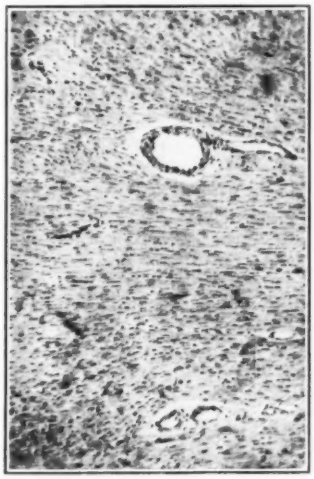

FIG. 2.—Myocardium of normal rabbit for comparison with Fig. 1 (same magnification).

This marking system was not absolutely necessary in the assessment of these results since there was not the slightest suggestion that any protection was given against the development of any of the observed lesions either by neoantergan or benadryl in the doses used.

Moderate or severe lesions were found in all 6 animals given neoantergan and in 5 of 6 animals given benadryl. The results are expressed in detail in the Table and Fig. 3.

TABLE

SEVERITY OF MYOCARDIAL LESIONS IN SERUM-SENSITIZED ANIMALS

Points were awarded for the presence of certain observed types of lesion. No protection is afforded by benadryl or neoantergan

Injections	No. of rabbits	Points	
		Total	Mean ± S.E.
Control serum only ..	10	26.5	2.65 ± 0.62
Serum plus Neoantergan, 5 mg./kg., s.c., b.d. ..	6	24	4.0 ± 0.86
Serum plus Benadryl, 10 mg./kg., s.c., b.d. ..	6	16.0	2.7 ± 0.64

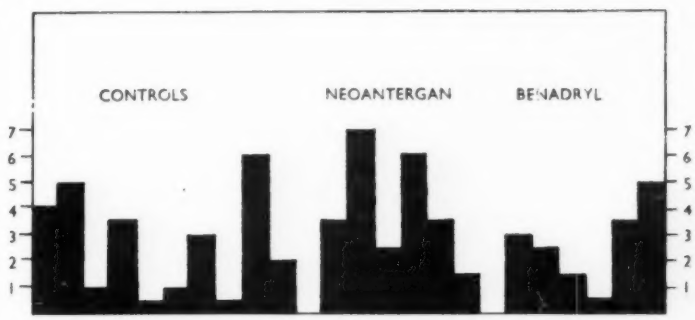

FIG. 3.—Showing the individual variation in the severity of the lesions produced in control and treated groups. Each column represents one animal and the ordinates are the points awarded to each rabbit (see text).

There was no alteration in the electrocardiographic pattern in either the control or treated group.

DISCUSSION

The histamine antagonists probably do not prevent the liberation of histamine during the antigen-antibody reaction (Bucher, 1948) but are able to antagonize the systemic effects of the liberated histamine. It is possible that antihistamine substances might prevent the pathological effects of serum disease and might consequently be of use in

treatment of diseases in which hypersensitivity is believed to play a part.

Some evidence has been reported that experimental nephritis in rabbits can be prevented by antihistamine substances (Reubi, 1946), and there have also been reports of the clinical usefulness of anthisan in human nephritis (Craig *et al.*, 1949) and of benadryl in polyarteritis nodosa (Sutherland, 1948). Examination of the kidneys of the animals used in the present experiments suggests that no significant protection against renal lesions due to hypersensitivity was afforded by the antihistamine substances.

The exact application of the above results in clinical practice is in doubt, but, in so far as the experimental lesions represent the nearest approach to the lesions of rheumatic fever, the results do not support the use of antihistamine substances in the treatment of human rheumatic fever; nor do they suggest that there is likely to be any benefit from their use in the treatment of polyarteritis nodosa. Hunter and Dunlop (1948) have reported that two cases of polyarteritis nodosa treated with antihistamine drugs were not improved.

Sodium salicylate is used routinely in rheumatic fever. Coburn (1943) has suggested that sufficiently large doses of salicylate, giving a blood concentration of about 35 mg./100 ml., may actually prevent the development of the cardiac lesions of acute rheumatism. Sodium salicylate has recently been shown to prevent the experimental cardiac lesions in rabbits subjected to serum sickness (Smull *et al.*, 1948; Ehrich *et al.*, 1949). Our preliminary experiments with sodium salicylate support this finding. Dammin and Bukantz (1949) suggest that administration of nitrogen mustards can provide protection against the development of the lesions.

Summary

1. Contrary to published findings, neoantergan and benadryl did not prevent the development of myocardial lesions in rabbits sensitized to horse serum.

2. The results do not support the therapeutic use of antihistamine substances in the treatment of rheumatic fever or of polyarteritis nodosa in man.

We wish to thank Professor E. J. Wayne for his help and encouragement in this work, Mr. E. Salvin for invaluable technical assistance including the histological preparations, and Mr. J. Kugler for the photomicrographs. The sterile horse serum used in these experiments was kindly supplied by Mr. G. J. Christie of Boots Pure Drug Co., Ltd. The expenses of this work were met by a grant from the University Research Fund.

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